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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02320 04 CHB

## PERIOD COVERED

October 1, 1986-September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacological Manipulation of HbF Synthesis

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Arthur W. Nienhuis, Chief, Clinical Hematology, CHB, NHLBI

Others: Brian Agricola, Animal Technician, Section on Animal Surgery

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Joseph E. Pierce, D.V.M., Chief Section on Animal Surgery,

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## COOPERATING UNITS (If any) Laboratory of Chemical Biology, NIMDDK.

Griffin Rogers, M.D. and Alan Schechter, M.D.

LAB/BRANCH Clinical Hematology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These studies are designed to determine, in experimental animals and humans, the ability of various substances to induce HbF synthesis. Several drugs have been shown to have this potential. Because of its safety and demonstrated activity, we have initiated a expanded phase I-II study of Hydroxyurea. The purpose of this study is to further define the frequency and magnitude of response of individual patients and to identify significant toxicity.

During the past several years, various hematopoietic growth factors have been molecularly cloned and large amounts have been synthesized in vitro. Several factors are now available for clinical testing. Those with activity on the erythroid progenitor and precursor population have the potential for stimulating HbF synthesis. Studies in Rhesus monkeys have been designed to test these substances. To date we have learned that erythropoietin has a definite, although limited ability to initiate HbF synthesis.

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Project description:Objectives:

Patients with severe beta thalassemia or sickle cell anemia would benefit significantly if HbF production could be consistently augmented. In thalassemia patients, increased synthesis of gamma globin (found in HbF) compensates for the deficiency of beta globin production. The imbalance of globin synthesis, characteristic of this syndrome, is partially corrected by increased gamma globin synthesis. Reduction of intracellular HbS concentration by replacement with HbF reduces the polymerization potential of intracellular sickle hemoglobin and therefore reduces the sickling "propensity" of red cells from these individuals.

Small amounts of HbF synthesis are found in the blood of normal humans. The HbF is heterogeneously distributed among circulating red cells; it is restricted to a minority of red cells called F-cells. F-cells find their origin during erythropoiesis as progenitor cells become committed to forming erythroblasts that either do or do not synthesize HbF. Most HbF is synthesized in the earliest stage of erythroblast maturation (pro-erythroblast and basophilic erythroblast). Regulation of the individual globin genes occurs at the level of transcription. The gamma globin gene retain an active chromatin structure and clearly have the potential to be transcribed at least in a fraction of erythroblasts. Thus there are numerous "points of attack" for agents that are designed to augment HbF synthesis.

Several classes of substances have been found to augment HbF synthesis. The first of these, 5-azacytidine, appears to directly modify DNA by inhibiting methylation thereby increasing gene expression. Hydroxyurea apparently works by perturbing the cell cycle or by killing rapidly dividing cells thereby allowing "regeneration" from earlier progenitors, such earlier progenitors are thought to have a higher potential for generating erythroblasts that synthesize HbF. Physiological regulators of hematopoiesis such as erythropoietin, interleukin 3 or granulocyte monocyte-colony stimulating factor have a definite but largely unexplored potential for augmenting HbF by perturbing erythroid progenitor and precursor kinetics.

Methods:

Drugs or hematopoietic growth factors are administered singly or in combinations to experimental animals. The hematological response to these substances is monitored by measuring the percentage of reticulocytes that contained HbF, the percentage of HbF containing red cells, and the percentage of HbF in peripheral blood. Patients are treated with hydroxyurea, orally. Blood levels are monitored and the parameters measured to monitor HbF synthesis are those noted above.



Major findings:

1. Erythropoietin has been shown to have a definite but limited ability to increase HbF synthesis in Rhesus monkeys. The response appears to be augmented by prior administration of a factor, such as GM-CSF, that acts on earlier erythroid progenitors.

2. Six patients have been treated with gradually escalating doses of hydroxyurea. The doses were chosen based on measurements of hydroxyurea absorption and clearance. Three patients have shown a response with a three to ten fold increase in HbF.

Proposed course of the project:

Our clinical trial of Hydroxyurea will be expanded to include additional patients. The frequency and dose of hydroxyurea will be adjusted based on accumulating data. Once the most effective regimen is defined, patients will be treated for periods of 3-6 months to define the maximum response. Concurrently, we intend to continue to explore the use of hematopoietic regulators to augment HbF synthesis in experimental animals. Major findings will be exploited by development of appropriate clinical protocols.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02321 03 CHB

## PERIOD COVERED

October 1, 1986-September 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Use of Antisense RNA or DNA to Inhibit Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:Robert L. Redner, M.D., Medical Staff Fellow, CHB, NHLBI

Others:Gail Osawa, Chemist, CHB, NHLBI

Elizabeth Rathbun, Medical Technologist, CHB, NHLBI

Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Hematology

## SECTION

## INSTITUTE AND LOCATION

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## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These studies are designed to analyze the effects of oncogene expression on cell growth and differentiation. The functions of the proto-oncogenes c-fos and c-myc will be studied by observing the phenotype of both benign (immortalized) and malignant cells when oncogene expression is inhibited. Recombinant DNA vectors designed to produce "antisense RNA" have been introduced into mouse fibroblast and embryonal teratocarcinoma cell lines. These vectors contain a steroid inducible (MMTV) or metal inducible (Metallothionein) promoter which should allow regulated production of the antisense RNA, or a Rous Sarcoma Virus LTR promoter for constitutive expression. Inhibition of c-fos or c-myc expression with these antisense constructs blocks proliferation of fibroblasts. Preliminary results suggest that antisense c-fos can also inhibit teratocarcinoma differentiation. Single stranded antisense DNA oligomers designed to anneal with c-myc mRNA inhibit HL-60 promyelocytic leukemia cell growth and induce early differentiation. Such selective inhibition of c-fos or c-myc expression may further elucidate the role of these proto-oncogenes in normal cellular growth and differentiation.

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Project Description:  
Objectives

Much evidence supports the concept that proto-oncogenes play key roles in both proliferation and differentiation of cells, and their deregulation or perturbation are often associated with malignancy. For instance, serum deprived mouse fibroblasts (3T3) exposed to serum factors respond with a 5-50 fold increase in c-fos mRNA, followed by c-myc expression and subsequent cell division. Human promyelocytic leukemia cells (HL-60) differentiate on exposure to phorbol esters; this differentiation is associated with an increase in c-fos and decrease in c-myc expression. However, the exact role that such proto-oncogenes play in cellular processes is not yet clear. The object of this project is to further characterize the significance of alterations in oncogene expression, by attempting to overcome normal proto-oncogene regulation via "anti-sense RNA" vectors. Anti-sense RNA technology is based on the observation that RNA complementary to mRNA will inhibit its expression, presumably by hybridization and translational arrest. If such anti-sense RNA vectors are driven by inducible promoters, the stable transformants could be specifically induced to down regulate proto-oncogene expression. Alternatively, antisense DNA oligomers can be used to transiently inhibit expression of a gene in a population of cells exposed to the oligomer. Any change in phenotype would thus implicate the proto-oncogene.

Methods

1. DNA vectors are constructed using standard recombinant DNA techniques and transferred into tissue culture cell lines with Calcium Phosphate precipitation. DNA and RNA are recovered from the cells by guanidinium extraction. DNA samples are analyzed by Southern blotting and hybridization to radiolabelled probes. RNA samples are analyzed by nuclease protection (S1 nuclease) or Northern blotting to quantitate the amount of specific RNA present in the cells. RNA probes for the antisense vectors have been developed in this laboratory. C-myc protein is quantitated by either immunoprecipitation or immunoblotting techniques.
2. Short single stranded oligomers are synthesized on an automated phase synthesizer and purified by high pressure liquid chromatography. These are added directly to cultures of HL-60 promyelocytes, and differentiation assessed by both morphologic criteria and the acquisition of the enzymes in the oxidative burst pathway.
3. Growth curves of 3T3 transformants in the presence and absence of the inducing agent will be performed, and the effects of growth factors on cells producing the antisense transcript constitutively will be analyzed.

Major Findings

3T3 stable transformants containing antisense-fos or antisense-myc vectors have been isolated. Induction of anti-fos RNA inhibits cell



proliferation almost completely. Antisense-myc clones show a similar, though less dramatic, decrease in proliferation, though clones producing the antisense-myc constitutively grow only when stimulated by growth factors. Studies have shown that antisense-fos vectors inhibit retinoic acid induced differentiation of F9 embryonal teratocarcinoma cells. Antisense-myc oligomers inhibit HL-60 promyelocytic growth, and induce early differentiation.

#### Proposed Course of the Project

The 3T3 antisense-myc clones and their response to growth factors will be further characterized. Antisense oligonucleotides will also be further investigated, as well as a variety of analogs less susceptible to degradation. Retroviral constructs containing the antisense sequences will be developed so as to broaden the range of cell lines that may be studied. The constructs will also be injected into mouse ova in an attempt to create a transgenic strain expressing the antisense RNA in an inducible manner. Collaborations with Dr. Ian Magrath's group in the NCI are also being established to study the effects of antisense-myc on Burkitt's Lymphoma cell lines.

#### Publications

1. Holt, J.T., et al., Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation. PNAS 83:4794, July 1986.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02324 03 CHB

## PERIOD COVERED

October 1, 1986-September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of Cis

and Trans-acting Elements that Regulate Human Gamma Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: David M. Bodine, Ph.D, Staff Fellow, CHB, NHLBI

Others: Arthur W. Nienhuis, Chief, Clinical Hematology Branch, NHLBI

Peter C. Hoppe, Ph.D, Senior Staff Scientist, The Jackson

Laboratory, Bar Harbor ME

Deborah Rothenhoefer, Biologist, CHB, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have been investigating the regulation of the human fetal  $\gamma$  globin genes to identify the transcriptional control signals for these genes. Among these transcriptional control elements are promoters, enhancer elements and inducible elements which act in cis to a gene, and trans acting factors that presumably control transcription and methylation.

Previously we showed that a 750 bp region located 3' to the  $A_{\gamma}$  gene had many of the properties associated with enhancer elements. We have completed our characterization of this sequence by demonstrating that this sequence increases mRNA transcription, and that it contains tissue specific DNA'se I hypersensitive sites. These findings confirm that this sequence has all of the characteristics commonly associated with enhancer elements.

We have also characterized in detail 3 lines of transgenic mice that contain linked human  $\gamma$  and  $\beta$  globin genes. We found that these genes were expressed either in the embryonic yolk sac ( $\gamma$ ) and fetal liver ( $\beta$ ) respectively. However the  $\gamma$  gene remains hypomethylated in the fetal liver even though it is not expressed, indicating that under methylation is necessary but not sufficient for transcription and that the factors that control transcription and methylation are not identical.

Finally, we have analyzed the function of all of the human globin gene promoters in the erythroid cell line K562, and found that short promoters are appropriately expressed, and that changes in the secondary structure of the  $\gamma$  promoter can cause an increase in transcription.

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Project Description:Methods:

Hybrid genes consisting of globin promoters of varying sizes were fused to the coding sequence of the chloramphenicol acetyl transferase (CAT) gene were generated by standard techniques. Likewise, the location of DNA'se I hypersensitive sites within the enhancer region were demonstrated by established methods.

To demonstrate that the  $\gamma$  enhancer causes an increase in properly initiated  $\gamma$ -CAT mRNA, 100 pica moles (pm) of  $\gamma$ -CAT or  $\gamma$ -CAT with the  $\gamma$  enhancer were transfected into  $10^7$  HeLa cells by  $\text{CaPO}_4$  coprecipitation. Forty eight hours post transfection, RNA was harvested from these cells and analyzed for properly initiated  $\gamma$ -CAT mRNA by  $S_1$  nuclease protection.

To analyze the expression of the human  $\gamma$  and  $\beta$  globin genes at different developmental stages in our transgenic mice, homozygous transgenic male mice were mated to normal female mice. The offspring of such a cross are all heterozygous for the human  $\gamma$  and  $\beta$  globin genes. At various times post mating, the embryos were harvested and RNA and DNA were extracted from the erythroid tissues. The expression of the human  $\gamma$  and  $\beta$  globin genes was analyzed simultaneously by  $S_1$  nuclease protection. The methylation of the trans genes was monitored by southern blot analysis using methylation sensitive restriction enzymes.

Studies of the globin promoters and the effects of point mutations on the  $\gamma$  promoter were performed in K562 cells using electroporation to introduce the various globin-CAT hybrid genes into the cells. Twenty  $\mu\text{g}$  of pRSVgpt (internal control) and 12 pm (the equivalent of 20  $\mu\text{g}$ ) of each test plasmid were cotransfected into  $2 \times 10^7$  K562 cells. Forty eight hours post transfection the cells were lysed by 3 cycles of freeze thaw and analyzed for gpt activity. Amounts of lysate containing equivalent amounts of gpt activity were subsequently analyzed for CAT activity.

Major Findings:

Analysis of RNA from HeLa Cells transfected with  $\gamma$ -CAT or  $\gamma$ -CAT with the enhancer demonstrated that the increase in CAT activity produced by the enhancer is caused by an increase in properly initiated  $\gamma$ -CAT mRNA. The increase in mRNA correlated precisely with the increase in CAT activity. In addition, K562 chromatin was shown to have 3 DNA'se I hypersensitive sites in the enhancer region, while non erythroid cell lines did not. These findings complete the characterization of the  $\gamma$  enhancer and demonstrate that a 750 bp region located 3' to the  $A_\gamma$  gene has all of the characteristics of a true enhancer element.

The expression of the linked human  $\gamma$  and  $\beta$  globin genes in our transgenic mice are similar to that seen by others analyzing unlinked genes, i.e.  $\gamma$  expression in the yolk sac (embryonic) and  $\beta$  expression in the fetal liver and marrow. Interestingly, the methylation of the  $\gamma$  globin gene has a more human pattern, remaining undermethylated in the



fetal liver, even though it is not expressed there, before becoming fully methylated in the adult marrow. This is a rare instance where a gene remains hypomethylated in a tissue where it is not expressed, and may indicate that there are separate factors controlling transcription and methylation.

The analysis of all 6 globin promoters ( $\alpha$ ,  $\zeta$ ,  $\epsilon$ ,  $G\gamma$ ,  $A\gamma$ , and  $\beta$ ) in K562 cells showed that the globin-CAT genes were expressed in transient assays in amounts consistent with the transcription (as measured by runoff transcription) and steady state levels of mRNA of their endogenous counterparts. This establishes that only minimal promoters (~ 400 bp) are required for appropriate expression. The continuing analysis of the HPFH mutations has shown that the mutant promoters give 5 fold higher expression than wild type only when supercoiled DNA is transfected. Transfection of linear DNA of either mutant or wild type leads to identical, lower levels of expression. This supports a theory that the difference in expression is caused by a change in the secondary structure of the DNA in the promoter region.

#### Proposed Course:

We intend to expand our transgenic mouse experiments, and to develop a transgenic mouse facility in our own laboratory. In addition we are examining the DNA's hypersensitivity of our trans genes during development to see whether it correlates with the expression of the human  $\gamma$  and  $\beta$  globin genes. Finally, we will attempt to locate binding sites for trans regulatory proteins in the  $\gamma$  enhancer region.

#### Publications:

1. Bodine, D.M., and Ley, T.J. (1987) An Enhancer Element Lies 3' to the Human  $A\gamma$  Globin Gene. EMBO J 6: (in press).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

701 HL 02327 02 CHB

## PERIOD COVERED

October 1, 1986-September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Effect of V-abl and IL3 Genes on Hemopoietic Stem Cell Differentiation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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others: Siu-Wah Chung, PhD, Fogarty Fellow, Lab of Genetics, NCI  
Sandra Ruscetti, PhD, Senior Investigator, Lab of Genetics, NCI  
Timothy M Browder, MD, Guest Worker, CHB, NHLBI  
David Bodine, PhD, Staff Fellow, CHB, NHLBI  
Cindy Dunbar, PhD, Medical Staff Fellow, CHB, NHLBI  
Arthur W. Nienhuis, MD, Chief, CHB, NHLBI

## COOPERATING UNITS (if any)

Lab of Genetics, NCI.

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

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National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Retroviral-mediated gene transfer provides an efficient means of introducing exogenous genes into hemopoietic cells. By this method, we have shown that the naturally occurring Abelson murine leukemia virus containing the v-abl oncogene can infect hemopoietic progenitor cells and lead to generation of tumorigenic hemopoietic cell lines. Recombinant retroviral vectors containing the IL3 (interleukin 3) and GM-CSF (Granulocyte-macrophage colony stimulating factor) have also been constructed and recombinant viruses generated. Infection of established cell lines, but not primary hemopoietic cells, with the IL3 virus resulted in acquisition of tumorigenicity. We have also shown that hemopoietic stem cells can be infected with the IL3 virus and when they were infused into lethally irradiated mice, a disorder similar to a myeloproliferative syndrome in humans was detected in some of these animals.



## Project Description

### Objectives

Our aim is to establish in vitro and in vivo systems in which stem cell behaviour can be monitored and manipulated. Introduction of the v-abl oncogene and genes coding for hemopoietic growth factors such as IL3 and GM-CSF into hemopoietic cells by means of retroviral-mediated gene transfer would offer interesting information as to the biological consequence of the infected cells.

### Methods:

Standard recombinant DNA technology was employed to generate various retroviral vectors containing IL3 and GM-CSF genes. In these recombinants, a neomycin-resistant selectable marker gene was also present. Recombinant DNA was then transfected into 2 virus packaging cells for the production of recombinant retrovirus. Selection of 2 cells that have taken up foreign DNA was done by scoring neomycin-resistant colonies. High titer virus stocks were then used to infect hemopoietic cells from various sources. Standard clonogenic stem cell assays were used to determine the frequency and types of progenitor cells infected by the viruses. In vivo studies were done by infusing the infected cells into syngeneic, lethally irradiated (900R) recipients. Day 14 spleen foci (CFU-S) from these recipients as well as those from the control were isolated, and analysed for presence and expression of the transferred genes. The malignant property of the infected cells was evaluated by injecting them into immunodeficient nude mice.

### Major findings:

We have shown that blast colonies rich in hemopoietic progenitor cells can be infected by Abelson virus and tumorigenic cell lines can be developed. We now have evidence that these progenitors can be as earlier as those at the level of multipotent progenitor cells. These results may have relevance to the pathogenesis of chronic myelogenous leukemia (CML).

Our studies of the IL3 virus indicate that endogenous factor production is insufficient to drive primary cells to reach the frankly neoplastic stage. By comparing infected primary cells and the established secondary cells, we conclude that additional genetic event(s) must have occurred in the secondary cells such that they are predisposed to progression to the frankly neoplastic stage once a factor-independent state is attained. We have now additional evidence that hemopoietic stem cells can be infected with the IL3 virus. Infusion of the infected cells into lethally irradiated recipient mice resulted in some animals having hematological disturbances such as increase in hematocrit, white cell count, organ enlargement and infiltration of hemopoietic cells correlated with the presence of factor-independent, neomycin-resistant hemopoietic progenitor cells.





In vitro studies of GM-CSF virus suggest that the expression and production of GM-CSF has a different biological consequence when compared with the results of the studies of the IL3 virus. No primary factor-independent cell lines can be obtained from cultures of cells exposed to GM-CSF virus and it appears that infection with GM-CSF virus cannot support formation of mixed colonies under standard condition. Infusing GM-CSF virus infected cells into lethally irradiated recipients resulted in development of pulmonary abnormalities.

Proposed course of the project:

More animals infused with GM-CSF virus are to be analysed to firmly established the in vivo effect of the infected progenitor or stem cells. Comparison of the IL3 and GM-CSF virus infected cells in lethally irradiated recipients and in genetically anemic W/W<sup>V</sup> recipients is also being done. To examine the question that factor-independence is due to an autocrine mechanism, a recombinant virus containing the IL3 cDNA with the signal peptide being deleted has also been generated.

References:

1. Wong PMC, Chung SW, Raefsky E, Eaves CJ and Nienhuis AW  
Blast colonies containing hemopoietic progenitor cells give rise to Abelson virus transformed cell lines. Expt.Hematol (In Press).
2. Wong PMC, Chung SW and Nienhuis AW  
Retroviral transfer and expression of the IL3 gene into hemopoietic cells. Genes & Development 1:358-365, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

701 HL 02328 02 CHB

## PERIOD COVERED

October 1, 1986-September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Modification of Retroviral Targeting via Hybrid Envelope Proteins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Other: Takashi Shimada, M.D., Ph. D., Visiting Associate, CHB  
John Abrahms, Ph.D., DNAX, Palo Alto, CA  
Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI  
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Clinical Hematology Branch

## SECTION

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## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These experiments are designed to test whether structural alterations of retroviral envelope glycoproteins can direct virus targeting for tissue-specific gene transfer. One avenue explores the expression of a hybrid envelope gene which encodes for a murine pluripotent colony stimulating factor, interleukin 3 (IL3). Such a virus would then specifically target bone marrow cells expressing the high affinity IL3 receptor (IL3R). A second avenue explores construction of a packaging line specific for CD4 lymphocytes to introduce HIV anti-sense genes. Both packaging lines should then allow tissue specific viral infection of therapeutic genes in vivo without the attendant risks of marrow transplantation.



## Project Description

### Objectives

Beta thalassemia, as well as other inherited genetic disorders would be treatable if the deficient genetic information could be supplied to the affected tissue and appropriate expression obtained. Retroviruses are efficient mediators for such gene transfer. In the murine system, efficient transfer has been obtained by exposing bone marrow cells in vitro to cells which produce retroviral vectors encapsidated in trans with ecotropic retroviral structural proteins. The procedure involves lethal irradiation of a syngeneic recipient and subsequent reinfusion of the infected bone marrow cells. If the glycoproteins on the retrovirus envelope could be manipulated so that infection would be specific for the target tissue for genetic insertion, gene transfer could then be attempted in vivo by viral infection. The advantages of such an approach would be to: (1) insure the tissue specificity of gene insertion thereby alleviating potentially damaging integrations into non-targeted tissues as well as abrogating the need for bone marrow transplantation, (2) molecularly targeting a membrane receptor of high affinity so that virus-ligand/cell-receptor interactions are not only tissue specific, but enhanced as well, and (3) covalent attachment of envelope components thus welding a sturdy virus for future concentration.

We are now exploring these ideas in two systems. One involves IL3, a murine pluripotent colony stimulating factor affecting survival as well as differentiation of bone marrow progenitors and precursors. IL3 receptor is exclusively expressed on bone marrow cells and more importantly stem cells, the actual repopulating cells targeted owing to their clonal expansion and self-renewal capabilities. The other system involves use of the specificity of the AIDS virus surface glycoprotein for the CD4 epitope on human helper T lymphocytes and macrophages.

### Methods

All of the packaging constructs described in last year's report have been constructed and analyzed. As will be described in the following section, none of the IL3 constructs eventually subserved packaging function. Remedies to be tested sequentially include:

- (1) an IL3/p15E recombinant processed via the native IL3 signal sequence. This gene has already been constructed and will be subcloned into an RSV expression vector. GAG and POL functions will be supplied by cotransfecting the construct into an envelope deletion mutant or into a purely xenotropic packaging line (which was produced in conjunction with last year's constructions).
- (2) deletion of the peptidase cleavage site within the previously constructed xenotropic recombinants so as to create a surface IL3/gp70-p15E fusion protein.
- (3) attachment of the IL3 sequences to the amino terminus of the xenotropic envelope gene thereby minimizing secondary and tertiary structural constraints on the gp70-p15E interaction.
- (4) further modification of the "bald virus" construct to include more native MoMLV sequences downstream of the signal peptidase cleavage site.



Moreover, the HIV envelope constructions are proceeding. One approach will utilize a hybrid HIV-MoMLV envelope recombinant which will encompass the exterior gp120/gp43 of HIV covalently attached to the transmembrane portion of MoMLV ecotropic p15E. Thus the exterior moieties will convey envelope specificity for CD4 while internally conserving ecotropic p15E-GAG interactions important for retroviral morphogenesis. GAG-POL functions will be subsumed by either an ecotropic or amphotropic ENV deletion mutant. A second approach will seek to recreate lentiviral packaging without the need for TAT or ART by driving the HIV GAG-POL and ENV genes off of heterologous promoters.

#### Major Findings

Analysis of the IL3 packaging lines described in last year's annual report revealed the following:

- There was no expression of the "bald virus" construct by IL3 immunofluorescence of glutaraldehyde or methanol fixed cells. This construct involved recreation of the signal sequence of MoMLV which should release translation and direct the nascent glycoprotein into the endoplasmic reticulum. The failure of expression may therefore be due to abortive translation. The constructs described above will attempt to bypass this error by using the intrinsic IL3 signal sequence or by including more MoMLV sequences downstream of the signal cleavage site.
- The insertion of IL3 sequences into a xenotropic envelope presented a different result. Even though polyglycine/serine encoding linkers were introduced to ameliorate the steric constraints on the hybrid molecule, the hybrid could never be detected on the surface even though methanol fixation revealed abundant expression intracellularly. Presumably although the recombinant envelope was synthesized and processed correctly, the non-covalent interactions of gp70 and p15E were disrupted such that surface transport allowed the hybrid to be excreted. The constructs described above will either attach IL3 to the extreme amino terminus and thereby diminish steric disruption or make the gp70-p15E attachment covalent by deleting the peptidase cleavage site.

#### Proposed Course

The new constructions described in methods are being pursued. Should IL3 or HIV surface immunofluorescence be accomplished, the next step would be to demonstrate specific infection in vitro by using a panel of receptor positive and negative cell lines as well as competitive inhibition by neutralizing IL3 antibody or blocking specific CD4 epitopes. Hopefully then, in vivo experiments could be conducted to assay the suitability of these packaging lines for a gene transfer protocol.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02330 01 CHB

## PERIOD COVERED

October 1, 1986-September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mapping of Hypertrophic Cardiomyopathy Locus

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Stephan E. Epstein, M.D., Cardiology Branch, NHLBI

Henry J. Lin, M.D., Clinical Hematology Branch, NHLBI

Barry J. Maron, M.D., Cardiology Branch, NHLBI

John J. Mulvihill, M.D., Clinical Epidemiology Branch, NCI

Dilys M. Parry, Ph.D, Clinical Epidemiology Branch, NCI

Ray White, Ph.D, Howard Hughs Med.Inst., Univ. of Utah, S.L.C., Utah

## COOPERATING UNITS (if any)

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Salt Lake City, Utah

## LAB/BRANCH

Clinical Hematology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The purpose of this protocol is to determine the chromosomal locus or loci of the genes responsible for hypertrophic cardiomyopathy (HCM). This heart disease is of obscure etiology and may range in its presentation from an incidental finding to sudden death. The gold standard in the diagnosis of HCM remains echocardiography. Approximately one-half of the cases are sporadic and one-half are familial. Previous family studies have suggested an autosomal dominant pattern of inheritance in familial cases. We have ascertained two large kindreds affected with the disease. An autosomal dominant pattern of inheritance is evident in both pedigrees. We are currently studying portions of both families with echocardiography while we collect blood for DNA extraction and blood protein polymorphisms determinations. This will allow us to use the technique of genetic linkage analysis to map the gene. This technique has been already used by others to map the genes responsible for diseases such as cystic fibrosis, Huntington's Chorea, Alzheimers disease and bipolar affective disorder. Once the gene has been localized questions of genetic heterogeneity can be addressed by studying patients outside of these two families. Ultimately the marker which establishes the chromosomal location can be used to obtain the gene itself.

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Project Description:Objectives:

The first objective of this project is to determine the chromosomal locus or loci of the genes responsible for hypertrophic cardiomyopathy. Identification of a marker linked to the gene for hypertrophic cardiomyopathy will yield immediate clinical applications and foster further basic research. Family members who carry the gene can be determined and questions of genetic heterogeneity can be addressed. Accurate assessment of penetrance can be made and questions pertaining to variable expressivity can be approached. The marker will also provide a method to clone the gene itself which ultimately will yield a better understanding of this condition.

Methods:

Gene mapping by linkage analysis is a technique that takes advantage of the behavior of chromosomes in germ cells during meiosis. Two loci close to each other on the same chromosome will not assort independently in subsequent generations. Therefore the process of mapping a gene by linkage is a search for known chromosomal markers which detect a pattern that is transmitted throughout a pedigree in association with the disease in question. These markers can be blood protein polymorphisms or localized fragments of DNA which differ between individuals (restriction fragment length polymorphisms). The former are often not helpful because of their limited diversity and restricted occurrence throughout the genome (approx 10%). Therefore although we will utilize blood protein markers, the bulk of our search will employ DNA polymorphisms that are detected by Southern blot analysis. Markers can be chosen so they are evenly spaced throughout the genome to provide a rough map in which the gene for HCM can be placed. Recently a set of probes which detect markers that are highly variable between individuals has become available. Using these probes will increase the chances of discriminating between homologous chromosomes and allow the tracking of first generation chromosomes as they are passed on to progeny. Analysis of the data will be accomplished by a computer program designed for this purpose.

Proposed Course:

At the present time we have collected blood from the two families under study and evaluated the members of one of them for the presence of the disease. We will soon be able to perform a linkage analysis using the blood protein polymorphisms from the first family. We are also ready to begin running Southern blots with DNA from both families using well characterized probes to detect DNA polymorphisms. Data from the first family can be immediately scored and analyzed while scoring of data from the second family will be done after their medical evaluations have been completed. Once linkage has been obtained questions mentioned in the objective section can be addressed. The isolation of the gene itself will require the use of techniques such as pulsed field gradient electrophoresis and cosmid cloning to screen the area in question for the putative gene. A recently developed technique by which very large segments of DNA can be cloned into yeast may prove to be very helpful as well.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02331-01 CHB

## PERIOD COVERED

October 1, 1986-September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Inhibition of HIV

Replication in T-Lymphocytes by Anti-sense RNA Sequences

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Takashi Shimada, Visiting Associate, CHB, NHLBI

Others: Bernhard Maier, Visiting Fellow, CHB, NHLBI

Hiroyuki Fujii, Visiting Fellow, CHB, NHLBI

Timothy Browder, Postdoctoral Fellow, CHB, NHLBI

Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

Hiroaki Mitsuya, Ph.D, Clinical Oncology Program, DCT, NCI

Samuel Broder, M.D., Clinical Oncology Program, DCT, NCI

## COOPERATING UNITS (if any)

Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute

## LAB/BRANCH

Clinical Hematology

## SECTION

Molecular Biology

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS

0.5

## PROFESSIONAL

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Acquired immunodeficiency syndrome is caused by a human immunodeficiency virus (HIV) that infects and destroys helper T-lymphocytes. These experiments are designed to test whether RNA sequences complimentary to portions of the HIV genome (anti-sense RNA) can block HIV replication or gene expression thereby creating T-lymphocytes that are resistant to the cytopathic affects of HIV. Segments of the HIV genome are placed in a reversed or anti-sense transcriptional orientation in a transcriptional unit that includes a strong promoter and appropriate RNA processing signals. Such anti-sense transcriptional units are introduced into T-lymphoid cell lines in vitro and expression of the anti-sense sequences documented by appropriate molecular analysis. Cell clones that express the anti-sense sequences at high levels will be tested to determine whether inhibition of HIV replication has been achieved. A systematic approach utilizing DNA fragments from various portions of the HIV genome under the control of several strong promoters is planned. If a effective anti-sense transcriptional unit is created, it will be incorporated into a recombinant retrovirus in an effort to facilitate transfer and expression in primary T-lymphocytes from infected patients.



Project Description:Objectives:

The immunodeficiency virus (HIV, or HTLV III) is the etiological agent for the acquired immunodeficiency syndrome (AIDS). This human retrovirus is highly trophic and cytopathic for CD4<sup>+</sup> helper T-lymphocytes. Infection is manifested by prolonged asymptomatic latency followed by severe depletion of CD4<sup>+</sup> cells and frequent opportunistic infections due to immunodeficiency. Because of the extreme morbidity, high mortality and epidemic proportions of the disease, development of effective preventive and treatment protocols is urgently required. Several therapeutic strategies have been proposed. Although much effort has been focused on development of a vaccine against HIV, progress has been slow in part due to the great genetic variability of the envelope protein. Much effort is directed toward drug development; an inhibitor of reverse transcriptase (3'-azido-3'-dioxypyrimidine or AZT) has been shown to have potent anti-viral activity in vitro and has already been used clinically with some success.

The work being conducted under this project is directed toward a different therapeutic strategy. The goal is to create in patients, populations of T-lymphocytes that are resistant to infection and the cytopathic effects of the HIV virus. RNA molecules complementary to the mRNA of a specific gene have been shown to inhibit expression of that gene at the protein level by blocking RNA processing, transport or translation. Such anti-sense RNA sequences might block replication of HIV by formation of RNA:RNA duplexes that inhibit reverse transcription. To adequately test the hypothesis that anti-sense sequences could be used to create a population of T-lymphocytes resistant to HIV infection, we plan a systematic scheme to test fragments from various parts of the HIV genome linked to strong promoters to insure high level expression. Our experiments will focus on portions of the HIV genome that are known to be involved in RNA processing or the initial steps of RNA translation as these steps have been shown to be susceptible to anti-sense RNA inhibition in other systems. In addition, certain fragments will be tested specifically for the ability to inhibit replication of the retroviral genome.

Methods:

1. Transcriptional units containing HIV sequences encoding anti-sense RNA will be created by conventional recombinant DNA techniques. To be utilized in many of the initial experiments is an expression vector developed in this laboratory (pLTN) that contains a dominant selectable marker (neo<sup>R</sup> gene) and globin RNA processing signals. Several strong viral or eucaryotic promoters will be individually linked to fragments of the HIV genome placed in a reverse transcriptional orientation upstream from the globin RNA processing signals. Control plasmids containing the HIV fragments in the correct transcriptional orientation will also be constructed. Recombinant plasmid molecules will be introduced into target T-lymphocytes (H9 cell line) by physical techniques





such as  $\text{CaPO}_4$  mediated transfer, electroporation or DEAE-dextran mediated transfer.

2. Construction of recombinant retroviruses for transfer of anti-sense transcriptional units: the most efficient available means for gene transfer is with retroviral vectors. Accordingly, potentially effective anti-sense transcriptional units will be inserted into two different retroviral vectors. The N2 retroviral genome contains the  $\text{neo}^R$  resistance marker and has been very useful in deriving high titer producer clones. A self inactivating vector that eliminates LTR driven transcribed sequences upon intergration and will also be explored as a vehicle for introducing the HIV anti-sense transcriptional units into target populations. Recombinant retroviral genomes will be introduced into ecotropic and then amphotrophic packaging lines. Producer clones that yield high titer supernatants will be identified by scoring neomycin resistant clones on both mouse fibroblast and human T-lymphocyte populations.
3. Targeted transfer of anti-sense transcriptional units into T-lymphocytes: as described in the project "Modification in retroviral targeting by hybrid envelope proteins"-(Z01 HL 02328 02 CHB), an effort is being made to create packaging cell lines that will generate retroviral particles containing specific proteins as part of the envelope. The concept is that such proteins can be used via ligand-receptor interaction, to target retroviral particles to particular cell populations. Naturally occurring HIV infection provides a model for this concept as the HIV envelope specifically interacts with the CD4 membrane protein that acts as a receptor in mediating viral entry. We propose to take advantage of this natural mechanism by utilizing the HIV envelope protein to carry anti-sense transcriptional units specifically into T-lymphocyte populations.
4. Testing of T-lymphocyte clones containing anti-sense transcriptional units: experimental and control T-lymphocyte clones will be exposed to HIV. Infection will be scored by immunological measurement of p24 gag protein or by immunofluorescent detection of retroviral proteins in cells. Furthermore, cells containing anti-sense transcriptional units that appear to inhibit viral propagation will be scored for the cytopathic affect of HIV.

#### Major Findings:

These experiments were initiated in June of 1987. To date several anti-sense transcriptional units have been created in plasmid gene transfer vectors. The initial constructions have focused on the region encoding the trans activating proteins, TAT and ART. H9 T-lymphocytes clones containing these anti-sense transcriptional units have been obtained and are being tested for their ability to withstand exposure to HIV.



Proposed Course:

There are two parts to our strategy. The first is to develop an appropriate anti-sense transcriptional unit and the second is to develop a retroviral vehicle to transfer this genetic element into target cells. We intend to test the possibility of using the HIV envelope protein to target specifically to T cells. If positive results are demonstrated in vitro, the high efficiency of retroviral mediated gene transfer would allow early extension to clinical trials.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02332 01 CHB

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Epstein-Barr Virus and Aplastic Anemia

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Bruce G. Baranski, M.D., Medical Staff Fellow, CHB, NHLBI

Others: Neal S. Young, M.D., Chief, Cell Biology Section, CHB, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

Cell Biology Section

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Epstein-Barr virus has been demonstrated clinically to precede some cases of aplastic anemia. Documentation of EBV infection was previously based on the clinical syndrome and viral serologies. We have undertaken a systematic search for EBV within the marrow of patients with aplastic anemia. Using a combination of immunofluorescence for viral antigens, in situ hybridization we have identified four patients with large quantities of EBV in their marrow but not peripheral blood. Using an in vitro hematopoietic colony assay, we have demonstrated that EBV is found within the erythroid colonies and is toxic to these colonies. Further studies are currently underway to elucidate the mechanism of toxicity to bone marrow.



## Epstein-Barr Virus and Aplastic Anemia

## PROJECT DESCRIPTION

## Objectives:

Multiple clinical observations and research findings indicate that viruses may be the pathogenic agent in some cases of aplastic anemia. Clinically, viral prodromes are common before the onset of aplastic anemia and non-a non-b hepatitis is commonly seen at the time of diagnosis. Research into the pathophysiology of aplastic anemia in this lab and others has shown that immunological changes seen in patients with aplastic anemia (inverted helper/suppressor ratios, activated T lymphocytes, and abnormal lymphokine production) are very similar to changes seen during viral infections.

Clinically, Epstein-Barr virus (EBV) commonly associated with aplastic anemia. Documentation of EBV infection preceding aplastic anemia was previously based on the clinical syndrome and viral serologies: to date, no systematic evaluation of the role of the virus in aplasia has been undertaken. Following the presentation of a patient with aplastic anemia one month after an acute EBV mononucleosis we have identified a total of four patients with large quantities of EBV in their bone marrow. We are also using an in vitro hematopoietic colony culture system to study the interaction of EBV with cells of the bone marrow and better understand the mechanisms of bone marrow suppression following EBV infection.

## Methods:

We have used four techniques to demonstrate EBV within the bone marrow of patients with aplastic anemia: immunofluorescence, Southern hybridization, *in situ* hybridization, and viral culture. Control samples included patients with acute mononucleosis, other patients with aplastic anemia, and normal individuals.

For in vitro studies, normal bone marrow was infected with EBV following removal of B lymphocytes- a known target cell of EBV infection. Cells were then cultured in methylcellulose medium with the necessary growth factors and colonies were isolated after 14 days. The colonies were enumerated, assayed for residual B cells, and multiple techniques (as above) were used to document the presence of EBV within the isolated colonies.

## Major findings:

We have identified three patients prospectively and one patient retrospectively who have large quantities of EBV





within their marrow but not in peripheral blood taken simultaneously. All patients had a recent clinical syndrome consistent with EBV infection, and viral serologies confirmed that all patients had a recent EBV infection. EBV was not identified in the marrow of normal individuals, other patients with aplastic anemia, and in individuals with acute mononucleosis. The patient identified retrospectively was one of forty patients whose marrow was stored and screened with Southern analysis. Viral cultures were obtained on the three patients identified at the time of diagnosis and the viruses isolated are currently under study.

In vitro, infection of normal bone marrow with EBV appears to decrease both myeloid and erythroid colony formation. With the B-958 virus (a commonly used isolate), a decrease of 30% in erythroid colony number and a decrease of 15% in myeloid colony number is commonly seen. Using a variety of techniques, we have identified EBV within the erythroid colonies, suggestive that EBV may infect hematopoietic progenitor cells.

#### Proposed course:

Based on the findings we have to date and known immunologic changes induced by EBV, we have postulated that EBV may induce bone marrow aplasia by a number of mechanisms. Firstly we have shown that EBV may infect hematopoietic progenitor cells directly, resulting in direct toxicity to stem cells. Further studies planned at this time include identification of the cell type infected by surface marker analysis. Other studies on T-cell cytotoxicity, antibody dependent cytotoxicity, lymphokine involvement, and accessory cell requirements for bone marrow toxicity are currently underway.



ANNUAL REPORT OF THE  
LABORATORY OF EXPERIMENTAL ATHEROSCLEROSIS  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE  
October 1, 1986 through September 30, 1987

Atherosclerosis is the underlying basis of most coronary artery disease, the leading cause of death in the United States. Because pathological cholesterol accumulation is central to the development of the atherosclerotic lesion, we have chosen to study this aspect of atherosclerosis.

At present, many mechanisms for the pathogenesis of atherosclerosis have been proposed. One research project in our laboratory has been directed towards identifying how platelets, through the release of lipid-rich particles, could play a role in the accumulation of lipids within cells that organize thrombi associated with atherosclerotic lesions. Previous work in our laboratory has shown that platelets can mediate cholesterol accumulation in cultured smooth muscle cells and other investigators recently have shown that platelets can mediate cholesterol accumulation in macrophages.

Specifically, we found that increasing amounts of cholesterol and phospholipid were continuously released from human or rat platelets that were activated with thrombin and incubated for a period of up to 2 hrs. Cholesterol release from platelets was also stimulated by strong platelet agonists such as collagen and the calcium ionophore A23187 but not by the weak agonist ADP. Colchicine substantially inhibited cholesterol release. The cholesterol and phospholipid that was released from platelets was contained within particles that were spherical or irregular in shape with sizes ranging from 50 to 550 nm. The particles had between 20 to 240 peripheral 18-nm long, rod-shaped projections extending from a bilayered membrane surrounding an inner core. The inner core of these particles contained either a granular or filamentous content. The filamentous content resembled actin microfilaments. In this regard, the major protein of the particles had an apparent molecular weight of 42,000 consistent with that of actin. Other chemical analysis showed that the particles contained 8% cholesterol, 32% phospholipids, 3% triglycerides, and 57% protein with a cholesterol to phospholipid molar ratio of approximately 0.6, a value typical for the plasma membrane. The ratio of cholesterol to phospholipid for particles from platelets of rats fed a high-cholesterol diet increased to 1.0.

The chemical and morphological data suggest that the released particles are formed as a result of vesiculation of the platelet plasma membrane. Because platelet activation is associated with microfilament formation extending into platelet pseudopods, pinching off of plasma membrane in pseudopod regions could give rise to those membranous particles that we observed with a microfilament content. Pinching off of plasma membrane in non-pseudopod regions could give rise to those membranous particles which we observed with a granular cytoplasmic content. Such a process of plasma membrane (exo)vesiculation may be a more generalized cell biological phenomenon as a similar process has been described in other cell types. We have now identified platelet-derived, cholesterol-phospholipid-particles released from activated platelets that may be important not only in coagulation but also in atherosclerosis.



We have now also completed studies of the isolation and characterization of a unique cholesterol-containing particle that we previously identified within atherosclerotic lesions. Purification of these particles was accomplished with microfiltration, gel filtration chromatography and density gradient centrifugation. Particles had a high percentage of their cholesterol in an unesterified form ( $\approx 75\%$ ) and a high molar ratio of unesterified cholesterol to phospholipid ( $\approx 2.5$ ). The average density of the particles was 1.036 g/ml. The particles were generally spherical, unilamellar vesicles with diameters ranging between 700 to 3000 Å. Freeze-fractured particles had smooth fracture faces lacking intramembranous particles distinguishing the membrane of these particles from cellular membranes. Upon incubation with filipin, the particles showed typical filipin-sterol complexes, demonstrating the presence of unesterified cholesterol in the vesicular membrane of these particles. No particles were found in aortas lacking atherosclerosis, further demonstrating the specificity of these vesicular cholesterol-phospholipid particles for atherosclerotic lesions.

We believe that the aortic particles we have isolated are possibly a degradation product of plasma low density lipoprotein (LDL) that has infiltrated into the vessel wall. Because the aortic lipid particles are about 5 times larger than LDL and because 75% of cholesterol in the aortic lipid particles is unesterified in comparison to LDL in which 80% of cholesterol is esterified, we suggest that if the aortic particles are derived from plasma LDL, they must be extensively altered, possibly as a result of cell-mediated processes within the vessel wall. Specifically, it would appear that the cholesteryl ester content of the LDL particle is hydrolyzed to unesterified cholesterol and free fatty acids. Consistent with the possibility that the aortic lipid particles are degradation products of LDL is our finding that a substantial amount of free fatty acids are associated with the aortic particles.

To examine the possibility that the unesterified cholesterol-rich aortic particles are derived from degraded esterified cholesterol-rich plasma LDL, we have carried out in vitro enzymatic digestions of the LDL particle. Interestingly, we have found that enzymatic hydrolysis of the cholesteryl ester core of the LDL particle does not occur without prior hydrolysis of the outer protein coat of the particle. Enzymatic hydrolysis of the cholesteryl ester core of the LDL particle reaches a maximum resulting in approximately 36% of the cholesterol remaining esterified. Digestion of the LDL cholesteryl ester results in changes in the structure of the LDL particle. LDL is normally a sphere with a diameter of 220 Å. Hydrolysis of the cholesteryl ester core results in the LDL particle changing shape to a larger vesicular particle similar to the aortic lipid particles we have isolated from atherosclerotic vessels. Enzymatic digestion of the LDL protein content alone is not sufficient to bring about this structural change. The results demonstrate that it is possible to generate, at least in vitro, a degradation product of plasma LDL that resembles in many respects the unique lipid particles that we have isolated from atherosclerotic vessels. Future experiments will be directed at examining whether cellular degradation of plasma LDL also results in a degradation product similar to the aortic lipid particles.



Atherosclerosis is a complex process of abnormal lipid deposition. Many factors presumably influence the pathogenesis, such as high plasma cholesterol levels and transport of plasma lipoproteins into the vessel wall. The unique unesterified cholesterol-rich particles we have isolated from atherosclerotic vessels constitute an early pathologic form of accumulated cholesterol in developing lesions. These particles may be a degradation product of infiltrated plasma LDL. Because these aortic particles appear prior to the formation of foam cells and progressively accumulate during the development of atherosclerotic lesions, we believe that they play an important role in the pathogenesis of atherosclerosis. Continued studies of the origin and fate of these unique unesterified cholesterol-rich lipid particles may contribute to a better understanding of the pathogenesis of the atherosclerotic disease process.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02826-06 EA

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Isolation and characterization of lipid-rich particles in atherosclerotic lesions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: F.-F. Chao Visiting Fellow LEA, NHLBI

Others: H. S. Kruth Senior Investigator LEA, NHLBI

## COOPERATING UNITS (if any)

Laboratory of Cellular & Developmental Biology, NIADDK (L. Amende and E. J. Blanchette-Mackie); Section on Laboratory Animal Medicine and Surgery, NHLBI; Department of Pathology, Univ. of Md. School of Medicine (J. Resau & W. T. Mergner)

## LAB/BRANCH

Laboratory of Experimental Atherosclerosis

## SECTION

Vascular Physiology Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this work was to study the chemical and structural properties of unique unesterified cholesterol-rich lipid particles isolated from the atherosclerotic lesions of human and rabbit aortas. These lipid particles were purified from aortic extracts by microfiltration, gel filtration and density gradient ultracentrifugation.

The purified lipid particles had a hydrated density between 1.02 g/ml and 1.08 g/ml with a peak at 1.036 g/ml. The lipid particles had a high molar ratio of unesterified cholesterol to phospholipid (2.4:1 in rabbit, 2.6:1 in human) and a high percentage of their cholesterol in an unesterified form (82% in rabbit, 76% in human). Particles from both human and rabbit aortas were similar in size and shape. They had a modal diameter of 1000-1200 Å (size range between 700-3000 Å) and appeared singly or in aggregates with uni- and multi-lamellar structures sometimes containing a non-aqueous core. Their fracture faces were smooth and devoid of intramembranous particles, indicating the membranes of these particles were not cellular membranes. Upon incubation with filipin, the particles showed typical filipin-sterol complexes, demonstrating the presence of unesterified cholesterol.

Glycosaminoglycan (GAG) were examined as a cause of the tendency of the lipid particles to aggregate. Incubation of lipid particles with chondroitinase ABC successfully digested the GAG content of the particles but did not appear to change the degree of particle aggregation. This suggests that GAG may not be a major factor mediating aggregation of the lipid particles.

A substantial amount of free fatty acids was found associated with the lipid particles (8% of total lipid, molar basis). The presence of such amounts of free fatty acids is consistent with the possibility that these particles might be a degradation product of plasma lipoproteins.

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## Project Description

Objectives: In work reported last year (Z01 HL 02826-05 EA), we isolated and purified unesterified cholesterol-rich lipid particles which we previously identified using histochemical techniques in the atherosclerotic lesions of human and rabbit aortas. In this year's project, we completed investigation of the chemical and structural properties of these lipid particles.

Methods: Unesterified cholesterol-rich lipid particles were extracted from aortic intimal-medial tissues with 8 volumes (w/v) of 0.15 M NaCl (pH 7.4) containing 0.1% EDTA, 0.05% glutathione and 0.02% NaN<sub>3</sub>. This lipid-rich extract was further purified by microfiltration (passed through a 0.45  $\mu$ m filter to remove lipid droplets and crystalline lipid) and dialyzed against a high salt solution (1.5 M NaCl/0.1% EDTA/0.05% glutathione/0.02% NaN<sub>3</sub>, pH 7.4) in order to help dissociate any lipid aggregates. The sample was passed through a Bio-Gel A-50m column which was eluted with the same high salt solution. Lipid particles that eluted in the void volume of the column were further purified with NaCl density gradient ultracentrifugation (continuous over  $d=1.006$  to  $1.100$ ) at 170,000 xg for 22 hrs at 4°C. Purified particles were analyzed for protein, unesterified and esterified cholesterol, phospholipid, triglyceride, free fatty acid and glycosaminoglycan. The structure of the lipid particles was examined by electron microscopy after the particles were negatively stained or freeze fractured. A portion of the sample was incubated with filipin (at a final concentration of 1.25 mg/ml) prior to negative staining or freeze fracturing to localize unesterified cholesterol.

Major Findings: The hydrated density of these particles was between 1.02 g/ml and 1.08 g/ml with a peak at 1.036 g/ml. The lipid particles had a high molar ratio of unesterified cholesterol to phospholipid (2.4:1 in rabbit, 2.6:1 in human) and a high percentage of their cholesterol in an unesterified form (82% in rabbit, 76% in human). Particles from both human and rabbit aortas were similar in size and shape. They had a modal diameter of 1000-1200 Å (size range between 700-3000 Å). These particles appeared singly or in aggregates with uni- and multi-lamellar structures. Their fracture faces were smooth and devoid of intramembranous particles, indicating the membranes of these particles were not cellular membranes. Upon incubation with filipin, the particles showed typical filipin-sterol complexes, demonstrating the presence of unesterified cholesterol.



Because the isolated lipid particles were often aggregated, we examined the possibility that this aggregation was mediated by glycosaminoglycan (GAG). The particles contained 56  $\mu\text{g}$  GAG/g of tissue which compared with 1250  $\mu\text{g}$  of lipid and 373  $\mu\text{g}$  of protein per g of tissue. Removal of this GAG with chondroitinase ABC digestion did not appear to alter the lipid composition or density distribution of the particles. However, although >98% of the GAG content was digested, there did not appear to be a change in the degree of particle aggregation. This suggests that GAG may not be a major factor mediating the aggregation of the lipid particles. Interestingly, a high cholesterol-phospholipid molar ratio has been shown to promote aggregation of lipid particles in model systems and may partly explain the aggregation we observed.

The free fatty acid concentration of the purified particles accounted for 8% of the total lipid (molar basis). The presence of such substantial amounts of free fatty acids and unesterified cholesterol is consistent with the possibility that these lipid particles might be a degradation product of plasma lipoproteins, especially low density lipoproteins.

Significance to Biomedical Research and the Program of the Institute:

Atherosclerosis is a complex process of abnormal lipid deposition. Because the particles we have isolated appear prior to the formation of foam cells and progressively accumulate during the development of atherosclerotic lesions, we believe they constitute an early pathologic form of accumulated cholesterol in developing lesions. Studies of the formation and fate of these unique unesterified cholesterol-rich lipid particles may contribute to a better understanding of the atherosclerotic disease process.

Proposed Course: This project has been completed. A new project has been initiated to examine the hypothesis that the aortic particles represent degraded plasma low density lipoproteins (see project Z01 HL 02829-01 EA).



Publications:

1. Kruth, H.S., Cupp, J.E., Khan, M.A., Henderson, G.R., Berlin, E., and Campbell, G.: Quantification of Cholesteryl Ester Containing Cells From Swine Aortas With Spontaneous and Experimentally Induced Atherosclerosis. In Tumbleson, M.E. (Ed.): Swine in Biomedical Research. Plenum Publishing Corp, 1986, pp. 1567-1572.
2. Kruth, H.S., Comly, M.E., Butler, J.D., Vanier, M.T., Fink, J.K., Wenger, D.A., Patel, S., and Pentchev, P.G.: Type C Niemann-Pick disease. Abnormal metabolism of low density lipoprotein in homozygous and heterozygous fibroblasts. J. Biol. Chem. 261: 16769-16774, 1986.
3. Kruth, H.S., Cupp, J.E., and Khan, M.A.: Method for detection and isolation of cholesteryl ester-containing "foam" cells using flow cytometry. Cytometry 8: 146-152, 1987.
4. Cupp, J.E., Campbell, G., Khan, M.A., and Kruth, H.S.: Flow cytometric quantification of cholesteryl ester-containing "foam" cells. I. Analysis of aortas from normolipidemic swine. Exp. Mol. Path. 46: 40-51, 1987.
5. Cupp, J.E., Khan, M.A., Campbell, G., Henderson, G., and Kruth, H.S.: Flow cytometric quantification of cholesteryl ester-containing "foam" cells. II. Analysis of aortas from cholesterol-fed swine. Exp. Mol. Path. 46: 52-63, 1987.
6. Kruth, H.S.: Accumulation of unesterified cholesterol in limbal cornea and conjunctiva of rabbits fed a high-cholesterol diet. Detection with filipin. Atherosclerosis 63: 1-6, 1987.
7. Sprecher, D.L., Kruth, H.S., Pierce, J.E., Lakatos, E., and Papadopoulos, N.: A familial basis for the heterogeneity in coronary atherosclerotic disease. Atherosclerosis 65:167-172, 1987.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 02828-02 EA
PERIOD COVERED October 1, 1986 through September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Characterization of cholesterol-containing vesicles released from platelets		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	S. Skarlatos	Staff Fellow LEA, NHLBI
Others:	H. S. Kruth	Senior Investigator LEA, NHLBI
COOPERATING UNITS (if any) Department of Transfusion Medicine, CC Section of Laboratory Animal Medicine and Surgery, NHLBI		
LAB/BRANCH Laboratory of Experimental Atherosclerosis		
SECTION Vascular Physiology Section		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The purpose of this project was to examine the release of cholesterol and phospholipid from activated human and rat platelets and to examine the chemical and structural characteristics of the released particle containing these lipids. Increasing amounts of cholesterol and phospholipid were continuously released from platelets activated with thrombin and incubated for a period up to 2 hrs. Cholesterol release from platelets was also stimulated by strong platelet agonists such as collagen and the calcium ionophore A23187 but not by the weak agonist ADP. Colchicine substantially inhibited cholesterol release.</p> <p>The cholesterol and phospholipid that was released from platelets was contained within particles that were spherical or irregular in shape with sizes ranging from 50 to 550 nm. The particles had between 20 to 240 peripheral 18-nm long, rod-shaped projections per particle extending from a bilayered membrane surrounding an inner core.</p> <p>Chemical analysis showed that these particles contained 8% cholesterol, 32% phospholipids, 3% triglycerides, and 57% protein. The cholesterol to phospholipid molar ratio of the particles was about 0.6 (a value typical for plasma membrane), whereas, the ratio of cholesterol to phospholipid for particles from platelets of rats fed a high-cholesterol diet was 1.0. Polyacrylamide gel electrophoresis of particle proteins in SDS-containing buffer showed a major protein component with an apparent M.W. of 42,000, presumably actin.</p> <p>The data suggest that the released particles are formed as a result of vesiculation of the platelet plasma membrane. Cholesterol release did not appear to result from simple lysis of platelets since the release of lactate dehydrogenase (a measure of lysis) and cholesterol could be dissociated. We have now identified platelet-derived, cholesterol-phospholipid particles that may play a role not only in coagulation by providing phospholipid but also in atherosclerosis by providing a source of cholesterol.</p>		



## Project Description

Objectives: The purpose of this project was to characterize the release of cholesterol and phospholipid from activated platelets and to examine the chemical and structural characteristics of the released particle containing these lipids.

Methods: Washed rat platelets were prepared from citrated (3.2%) blood removed with a 19 gauge needle from the abdominal vein of ether-anesthetized, 3-month-old male rats (Sprague-Dawley). Washed human platelets were prepared from fresh human acid citrate/dextrose-treated blood obtained from fasting healthy donors by plateletpheresis. In both cases platelet-rich plasma was centrifuged for 30 min at 1400 xg. The supernatant was removed, and platelet pellets were resuspended in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free Dulbecco's phosphate-buffered saline (DPBS) containing 1% glucose and  $\text{PGE}_1$  or  $\text{PGI}_2$ . The platelet suspensions were rinsed twice in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free DPBS containing 1% glucose and  $\text{PGE}_1$  or  $\text{PGI}_2$ . Platelets were counted and rinsed a final time. The washed platelets were resuspended in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free DPBS with 1% glucose and added to a 150-mm tissue culture dish at a concentration of  $60 \times 10^8$  platelets per milliliter of Eagle's minimal essential medium.

Platelets that were to be activated received 1 unit/ml of human thrombin or 0.1 mg/ml of collagen. Incubations were typically carried out for 2 hours. After incubation, medium was harvested from each dish, pooled and centrifuged. The supernatant was then passed through a  $0.8 \mu\text{m}$  filter in order to remove any remaining platelets. The platelet-free supernatants were applied to a Sephadex CL-4B gel filtration column in order to isolate the cholesterol-containing moiety released by the platelets. Collected fractions were assayed for protein, cholesterol, phospholipid, and triglyceride. In addition, lipid particles that eluted in the void volume fraction were negatively stained or freeze fractured and examined with an electron microscope. Lipid particles were also treated with filipin to localize cholesterol by electron microscopy. The density of the lipid particles within the void volume fraction was determined by density gradient centrifugation in a continuous linear sucrose gradient.

Major Findings: Increasing amounts of cholesterol and phospholipid were continuously released from platelets for a period up to 2 hrs following platelet activation. With gel filtration chromatography all of the cholesterol and phospholipid released from activated platelets eluted in the void volume fraction. This suggested that the released cholesterol was associated with relatively large-sized particles. The platelet-derived particles demonstrated a density of 1.16 when centrifuged isopycnicly in a continuous sucrose density gradient. Electron microscopic analysis of particles released from activated platelets showed them to be spherical or irregularly shaped particles ranging in diameter



from 50 to 550 nm. The particles had between 20 to 240 peripheral 18-nm long, rod-shaped projections per particle extending from a bilayered membrane surrounding an inner core. The inner core was comprised of loosely packed electron dense granular or filamentous material. The long filamentous material (50 Å in diameter) resembled microfilaments and was also sometimes associated with the particle surface. Particles treated with filipin to localize cholesterol with electron microscopy contained characteristic filipin-sterol complexes located within the particle bilayer and not associated with the inner core. Analysis of rat particles visualized by negative staining demonstrated a modal size of 1000 to 1100 Å and a mean size of  $1550 \pm 67$  Å (SEM). Analysis of the human particle sizes showed a modal size between 2500-3000 Å with a mean size of  $2810 \pm 9$  Å (SEM). Much less cholesterol and far fewer particles were released from unactivated platelets. These particles were more spherical in shape (diameter of 160 nm) with approximately 50 peripheral projections per particle.

Chemical analysis of the gel filtration void volume fractions showed that particles released from activated human or rat platelets after 2 hrs incubation contained approximately 8% cholesterol, 32% phospholipids, 3% triglycerides and 57% protein. The cholesterol to phospholipid ratio of these particles was about 0.6. Particles released from platelets of rats fed a high-cholesterol diet were also studied. The percent cholesterol (16%) in these particles was twice that in normal particles whereas the percent phospholipid (34%) remained similar to that in normal particles. Thus, the molar ratio of cholesterol to phospholipid in particles released from activated platelets of rats fed a high-cholesterol diet was increased to 1.0.

Polyacrylamide gel electrophoresis of particle proteins in SDS-containing buffer (both unreduced and reduced samples) on 7% (w/v) polyacrylamide gels showed a major protein component of  $M_r$  42,000. This protein component most likely represents actin which has been shown previously to be associated with the platelet plasma membrane.

Although thrombin-activation of platelets did induce release of lactate dehydrogenase (a cytoplasmic marker) during the standard incubation period, it did not appear that release of cholesterol simply resulted from lysis of platelets. This was indicated by the observation that release of lactate dehydrogenase and cholesterol could be dissociated. Whereas colchicine (1 mM) substantially inhibited cholesterol release from thrombin-activated rat and human platelets (<15% of control), it only slightly decreased lactate dehydrogenase release (remained >80% of control).



Significance to Biomedical Research and the Program of the Institute:

The chemical and morphological data suggest that the released particles are formed as a result of vesiculation of the platelet plasma membrane. Because platelet activation is associated with microfilament formation extending into platelet pseudopods, pinching off of plasma membrane in pseudopod regions could give rise to those membranous particles that we observed with a microfilament content. Pinching off of plasma membrane in non-pseudopod regions could give rise to those membranous particles that we observed with a granular cytoplasmic content. Such a process of plasma membrane (exo)vesiculation may be a more generalized cell biological phenomenon as a similar process has been described in other cell types.

At present, many mechanisms for the pathogenesis of atherosclerosis have been proposed. Our research has been directed towards identifying how platelets, through the release of lipid-rich particles, could play a role in the accumulation of lipids within cells that organize thrombi associated with atherosclerotic lesions. Previous work in our laboratory has shown that platelets can mediate cholesterol accumulation in cultured aortic smooth muscle cells and other investigators recently have shown that platelets can mediate cholesterol accumulation in macrophages. We have now identified platelet-derived, cholesterol-phospholipid particles that may play a role not only in coagulation by providing phospholipid but also in atherosclerosis by providing a source of cholesterol.

Proposed Course: Project completed.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 HL 02829-01 EA
PERIOD COVERED October 1, 1986 through September 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structural change of human LDL induced by hydrolysis of its cholesteryl ester		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	F.-F. Chao	Visiting Fellow LEA, NHLBI
Others:	H. S. Kruth	Senior Investigator LEA, NHLBI
COOPERATING UNITS (if any) Laboratory of Cellular and Developmental Biology, NIADDK (L. Amende and E. J. Blanchette-Mackie)		
LAB/BRANCH Laboratory of Experimental Atherosclerosis		
SECTION Vascular Physiology Section		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1	PROFESSIONAL: 0.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) <p>             We believe that hydrolysis of low density lipoprotein (LDL) cholesteryl ester may be one of the mechanisms that contributes to the accumulation of unesterified cholesterol-containing vesicular particles in the vessel wall during atherogenesis (Z01 HL 02826-06 EA). The purpose of this project is to study the change in structure of the LDL particle when its oily cholesteryl ester core has been enzymatically hydrolyzed.           </p> <p>             Human LDL is a spherical lipid particle with size ranging between 20 to 25 nm. It has a surface layer formed with protein, phospholipid and unesterified cholesterol and a core that contains predominantly cholesteryl ester. The cholesteryl ester core of LDL was not hydrolyzed when incubated with cholesterol esterase alone at 37°C for up to 6 hrs. When LDL was treated with trypsin for up to 2 hrs to degrade its surface protein, the lipid composition and structure of the particle was also not changed. However, after treatment with trypsin, LDL cholesteryl ester became susceptible to hydrolysis by cholesterol esterase.           </p> <p>             Hydrolysis of cholesteryl ester resulted in a change in LDL structure. With 10 min of cholesterol esterase treatment, the hydrolyzed LDL appeared to be a somewhat flattened spherical structure which was slightly larger than unhydrolyzed LDL. After 2 hrs of cholesterol esterase treatment, the hydrolyzed LDL transformed into large flattened vesicles with irregular shape and size (60-300 nm), even though the lipid composition of the particles did not further change with the additional incubation time. These large vesicles contained unesterified cholesterol at their surfaces as detected by reaction with filipin. The results show that LDL can be transformed to a larger vesicular particle when its cholesteryl ester has been partially hydrolyzed. The hydrolyzed LDL particles resemble the unesterified cholesterol-rich lipid particles we have isolated from atherosclerotic vessels.           </p>		

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## Project Description

Objectives: The purpose of this project is to study the change in structure of the human low density lipoprotein (LDL) particle when its oily cholesteryl ester core has been enzymatically hydrolyzed by cholesterol esterase. We believe that hydrolysis of LDL cholesteryl ester may be one of the mechanisms that lead to the accumulation of unesterified cholesterol in the vessel wall during atherogenesis.

Methods: Human LDL (density range: 1.019-1.063 g/ml) was isolated from fresh human plasma containing EDTA-Na<sub>2</sub> and Na-citrate by sequential isopycnic ultracentrifugation at the lower and upper limit densities. The lipoproteins were washed with 1.063 g/ml solution by centrifugation and dialyzed against a 0.15 M NaCl and 0.01% EDTA-Na<sub>2</sub> (pH 7.4) solution. Twenty-five  $\mu$ l of human LDL (5 mg of protein per ml) was added to a solution containing 0.15 M NaCl, 5 mM Tris, and 0.5 mM EDTA-Na<sub>2</sub> (pH 7.2). Trypsin (45 U) was added to the LDL solution and the mixture was incubated at 37°C in a shaking water bath for 2 hrs. After this initial incubation, 25  $\mu$ l of soybean trypsin inhibitor (10 mg/ml) was added to neutralize the trypsin. Then, 5.5 U of cholesterol esterase (*Candida cylindracea*) was added and incubated under the same conditions for various times. The reaction was stopped by adding methanol and lipid was extracted by the method of Folch. Lipid extracts were assayed for esterified and unesterified cholesterol and phospholipid. Aliquots of unextracted samples were examined with an electron microscope. Samples were fixed at 4°C with equal volumes of 2.5% glutaraldehyde in phosphate-buffered saline for 30 min and negatively stained with 2% phosphotungstic acid.

Major Findings: Human LDL is a spherical lipid particle with size ranging between 20 to 25 nm. It has a surface layer formed with protein (predominantly apoB), phospholipid and unesterified cholesterol. Its core contains predominantly cholesteryl ester and small amounts of triglyceride. The chemical composition and structure of LDL was very stable. Incubation of LDL in 0.15 M NaCl/5 mM Tris/0.5 mM EDTA-Na<sub>2</sub> (pH 7.2) buffer at 37°C in a shaking water bath for up to 24 hours did not alter its lipid composition or structure. Incubation of LDL with cholesterol esterase for up to 6 hrs at 37°C did not hydrolyze its cholesteryl ester core. When LDL was treated with trypsin for up to 2 hrs to degrade its surface protein, the lipid composition and structure of the particle was also not changed. However, after treatment with trypsin, LDL cholesteryl ester became susceptible to hydrolysis with cholesterol esterase.



The time course of cholesteryl ester hydrolysis showed that LDL cholesteryl ester was linearly hydrolyzed from its initial 75% value (i.e., molar % of total cholesterol that was esterified) to 36% during the first 10 min. The amount of LDL cholesteryl ester did not decrease significantly further between 10 min and 2 hrs. Upon incubation of LDL with cholesterol esterase for greater than 2 hrs, a reverse reaction occurred in which re-esterification of cholesterol slowly proceeded. After 24 hrs of cholesterol esterase incubation, the degraded LDL contained 53% of its cholesterol as ester.

Hydrolysis of cholesteryl ester resulted in a change in LDL structure. After 10 min of cholesterol esterase treatment, the hydrolyzed LDL appeared to be a somewhat flattened spherical structure slightly larger than unhydrolyzed LDL. After 2 hours of incubation, the hydrolyzed LDL transformed into large flattened vesicles with irregular shape and size ranging between 60-300 nm. This occurred even though the lipid composition of the LDL particles did not further change during the additional incubation time. These large vesicles contained unesterified cholesterol at their surfaces as detected by reaction with filipin. The results show that LDL can be transformed to a larger and flatter particle when its cholesteryl ester has been partially hydrolyzed.

We have also examined factors that regulated the cholesteryl ester hydrolysis. In LDL treated with human serum albumin (final concentration of 1%, w/v) prior to the addition of cholesterol esterase, the cholesteryl ester hydrolysis was partially blocked. We have also observed that addition of sphingomyelin promoted cholesteryl ester hydrolysis.

#### Significance to Biomedical Research and the Program of the Institute:

Accumulation of cholesterol in the arterial vessels is a characteristic of atherosclerosis. In other work, we have observed that unesterified cholesterol-rich lipid particles accumulate in the extracellular space of the arterial wall during the development of atherosclerosis. Plasma LDL is known to be the major cholesterol carrier in the circulation and it is believed that LDL contributes significantly to the cholesterol deposition that occurs during atherogenesis. The relationship between plasma LDL and the unesterified cholesterol-rich particles we have isolated is still obscure. However, if LDL is a precursor of the unesterified cholesterol-rich lipid particle, its cholesterol ester-rich core must undergo extensive hydrolysis.

In this project, we studied the structural changes in plasma LDL induced by the hydrolysis of its cholesteryl ester core and explored the possibility that LDL could be a source of the unesterified cholesterol-rich lipid particles that we have isolated from atherosclerotic vessels. Our results indicate that hydrolysis of the LDL



cholesteryl ester core only began when the surface protein of the LDL particle was degraded. Hydrolysis of the cholesteryl ester in LDL was accompanied by a dramatic structural change in the LDL particle. After the cholesteryl ester had been partially hydrolyzed, the small LDL particles transformed into larger flattened vesicles with sizes ranging between 60-300 nm. Thus, the hydrolyzed particles resembled the unesterified cholesterol-rich lipid particles we have isolated from atherosclerotic vessels (Z01 HL 02826-06 EA).

Proposed Course: We plan to continue studies of the precursor-product relationship between plasma LDL and aortic unesterified cholesterol-rich lipid particles. We will explore the possibility that LDL is transformed to aortic unesterified cholesterol-rich particles by a cell-mediated process.





ANNUAL REPORT OF THE  
HYPERTENSION-ENDOCRINE BRANCH  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE  
October 1, 1986 through September 30, 1987

This year the Hypertension-Endocrine Branch has continued its basic and clinical research into many aspects of the causes and therapy of hypertension. These studies have focused on some of the neurohumoral and vasoactive systems that control the circulation.

I. Catecholamines and the sympathetic nervous system. The activity of the sympathoadrenomedullary system changes with nearly every activity in our lives, and these changes are vital for preserving the constancy of our internal environment. We face the challenge of understanding the pathophysiology of this system and the development of valid techniques to assess its activity. We have developed high pressure liquid chromatographic and electrochemical techniques to measure the catecholamines, epinephrine (E), norepinephrine (NE) and dopamine (DA), as well as dihydroxyphenylalanine (DOPA), dihydroxyphenylglycol (DHPG), and dihydroxyphenylacetic acid (DOPAC) in both blood and urine. We have improved our methods for measurement of total body and regional release of NE into the blood stream using infusions of tracer-labeled NE and isoproterenol (I). Using these techniques, we have shown that during the playing of a video game, blood pressure, pulse rate, cardiac output, and forearm blood flow are all increased. Antecubital venous levels of NE were unchanged, while arterial levels of NE increased significantly, and total body spillover of NE increased to a substantial extent. While changes in antecubital venous NE were unrelated to changes in systolic blood pressure or cardiac output during the game, changes in total body spillover of NE clearly were related to the circulatory changes. In the forearm, as blood flow increased, the clearance of NE increased linearly. The slope of this relationship was less than 1, however, because as the blood flow increased, the proportionate extraction of arterial NE decreased exponentially. As the cardiac output increased, the total body clearance of NE increased as well. In essence, the calculation of total body spillover of NE takes into account the blood flow dependence of NE clearance. Changes in antecubital venous NE can underestimate regional spillover of NE when forearm blood flow is increased, and changes in arterial NE can underestimate total body spillover of NE when cardiac output is increased. The use of the total body and regional spillover technique, therefore, appears to be a distinct improvement in the assessment of sympathetic activity and its hemodynamic consequences compared with antecubital venous levels of NE. When we began to apply the tracer infusion technique to patients 40 years old or younger with essential hypertension, we learned that hypertensives had normal rates of spillover of NE into arterial blood at rest and normal increments in total body spillover of NE during mental challenge. However, hypertensives had excessive increases in mean arterial pressure and total peripheral resistance to blood flow during mental challenge. These findings would suggest that vascular hyperresponsiveness to endogenously released NE occurs early in the development of essential hypertension since these patients were all 40 years old or younger. Application of the same technique to patients with hypertrophic cardiomyopathy has shown a surprisingly large amount of uptake-1 activity in the heart due to a large arterial venous increment in plasma DHPG, a much larger proportion of NE removal in the heart than I removal, and a



large amount of radioactive DHPG production by the heart during infusion of tracer-labelled NE. This is a very important finding because it would indicate that the heart is virtually completely dependent on uptake-1 to remove NE, and this may explain the susceptibility of the heart to cocaine toxicity, since cocaine releases NE and inhibits uptake-1.

We explored the origin of DHPG with studies in both man and rat. When alpha-2 adrenoreceptors were blocked with yohimbine, plasma NE, blood pressure and plasma levels of DHPG were increased, but when neuronal uptake of NE was blocked using desipramine, then yohimbine no longer increased DHPG, even though the NE and blood pressure responses were enhanced. Therefore, the increments in plasma DHPG caused by yohimbine were due to reuptake and intraneuronal metabolism of released NE. These findings provide the first clinical evidence that plasma DHPG derives from and can indicate intraneuronal metabolism of NE. After administration of clonidine to inhibit NE release, we found that plasma DHPG decreased to a plateau level of about 750 pg/ml, in contrast with plasma NE, which decreased to very low levels. Since plasma DHPG appears to derive from sympathetic nerve endings after metabolism of cytoplasmic NE, the most likely explanation for the continued production of DHPG when NE release was inhibited is leakage of NE from the storage vesicles into the cytoplasm. This leakage process can be quite active; thus, we found that in conscious rats, administration of reserpine markedly increased plasma DHPG as well as radioactive DHPG during administration of tracer-labelled NE. Thus increments in plasma DHPG during sympathetic stimulation reflect reuptake and metabolism of released NE back into the axonal cytoplasm, and when release of NE is small, then plasma DHPG indicates leakage and metabolism of stored NE from the vesicles into the cytoplasm. We have administered the alpha-2 adrenoreceptor antagonist yohimbine to young patients with essential hypertension and normotensive control subjects. About one-half of the hypertensive patients, but none of the controls, had marked hypertensive, sympathoadrenomedullary, and behavioral responses to yohimbine. This suggests that in a substantial portion of young patients with hypertension, alpha-2 adrenoreceptors in the brain control sympathetic outflow even during mild laboratory challenges, but when these receptors are blocked, then previously unrecognized increased sympathoadrenomedullary activity becomes apparent.

The source of plasma DOPA has been unclear. When we measured plasma DOPA levels in an artery and in the antecubital vein of the arm, the levels in the vein virtually always were higher. We have found that in patients who had undergone a regional sympathectomy, the concentration of DOPA in the arm vein was lower than in an artery. This provides strong clinical support for the notion that plasma DOPA can derive at least partially from sympathetic nerve endings. We also found that plasma DOPA levels were increased in patients with neuroblastoma or malignant pheochromocytoma, tumors of neural crest origin. Since DOPA is the product of hydroxylation of tyrosine, and since tyrosine hydroxylase is found exclusively in tissues which can synthesize catecholamines, we measured the effect on plasma DOPA of administration of the catecholamine synthesis blocker, alpha-methyl-paratyrosine. One day after a single dose of alpha-methyl-paratyrosine, we found that the plasma DOPA was decreased by 60%. This suggests that plasma DOPA derives to a substantial extent from catecholamine synthesizing tissues. Passive tilt, which rapidly increases plasma NE, has little effect on plasma DOPA in normal volunteers. General anesthesia decreases plasma DOPA by up to 75% in dogs and monkeys. In anesthetized dogs, prolonged ganglionic blockade decreased plasma DOPA, while



prolonged nitroprusside-induced hypotension increased plasma DOPA. These findings are consistent with the view that changes in plasma DOPA can reflect changes in tyrosine hydroxylase activity. Additional studies have suggested that as cytoplasmic NE increases, the amount of feedback inhibition of tyrosine hydroxylase increases, resulting in decreased production of DOPA and decreased plasma levels of DOPA. These data would suggest that circulating DOPA could provide a substrate for the creation of DA even in the cells which lack tyrosine hydroxylase, such as renal tubular cells. We have obtained evidence that in dogs at least one-half of urinary DA derives not from regional dopaminergic nerves, but from plasma DOPA. In proximal tubular cells of the kidney, DOPA is converted extensively to DA, and DA in turn inhibits sodium, potassium, ATPase, and stimulates natriuresis. We have obtained clinical evidence that during a high-salt diet, the excretion of DOPA doubled without a change in plasma DOPA. In a subject who received the DOPA decarboxylase inhibitor, carbidopa, urinary DOPA excretion increased by about 10-fold, while urinary DA excretion was cut in half. These findings would be consistent with the view that DOPA is a neurohormone which can inhibit sodium potassium ATPase directly, that DOPA continually is being released into the blood stream at a high rate and actively removed in the kidney, and that uptake of DOPA is enhanced during salt loading.

II. Atrial natriuretic peptides. We have developed sensitive radio-immunoassay procedures for measurement of atrial natriuretic peptides (ANP). These biologically active peptides are contained within specific secretory granules within mammalian atrial myocytes and possess potent natriuretic, diuretic and vascular smooth muscle relaxant properties. On the basis of extensive studies in rats, we have shown that rapid blood volume expansion produces a two- to three-fold increase of ANP in conscious animals. Hypophysectomy performed 1, 2, 8, or 30 days earlier produces a significant reduction in both basal and stimulated release of ANP. The concentrations of ANP in left and right atria of hypophysectomized rats were either normal or higher than that of the sham-operated animals. These results indicated that depleted stores in hypophysectomized rats were not the cause of the reduced release of ANP. Hypophysectomized animals into which the excised pituitary had been reimplanted under the renal capsule had normal levels of basal and stimulated ANP. This restoration of ANP release was dependent upon anterior pituitary auto-transplants and was not seen in autotransplants of either the neuro-intermediate lobe or posterior pituitary. Supplementation of hypophysectomized animals with prolactin, LH, FSH, and growth hormone did not restore ANP release. Furthermore, bolus administration of pharmacologic doses of drugs which modify circulating levels of prolactin such as bromocriptine or haloperidol also had no effect on basal or stimulated release of ANP. The plasma levels of ANP in the basal state and following an acute blood volume expansion are reduced during hypothyroidism, while the percentage of increase following blood volume expansion is unchanged. Hyperthyroidism does not alter basal or stimulated plasma levels of ANP, while acute TRH administration increases ANP levels in parallel with the increase in blood pressure, but does not alter the responsiveness to acute volume expansion. Angiotensin converting enzyme (ACE) and kallikrein have been reported to have effects on the metabolism of ANP. These two enzymes can be blocked by captopril and aprotinin respectively. Thus we studied the basal level of ANP, the response of ANP to acute volume expansion, and the level of ANP following an acute bolus of the peptide in rats with and without inhibition of these two enzymes. The combined inhibition of ACE with captopril and of kallikrein with



aprotinin reduced basal levels of ANP. The increase in ANP following an acute expansion of blood volume or IV injection of ANP was not altered by enzyme inhibition. Only a minor prolongation of the elimination of exogenous ANP was noted following inhibition of ACE alone, while no difference in blood pressure response, urinary volume, or sodium and potassium excreted was noted after any of the interventions. To learn more about the physiologic significance of ANP, baroreflexor sensitivity was determined before and after infusions of ANP to rats in doses that did not affect either basal blood pressure, heart rate, or the magnitude of the blood pressure response to either phenylephrine or nitroprusside. The bradycardic response to phenylephrine was accentuated and the baroreflex sensitivity increased by ANP. There was no significant effect of ANP on nitroprusside-induced changes in heart rate. Thus, in conscious rats ANP selectively enhanced the baroreflex response to increases, but not to decreases, in arterial pressure. In collaborative studies, we have measured plasma levels of ANP in children with congenital heart disease, tachycardia, altered blood volume, or critical illness. Results indicate that ANP in children mainly is produced by the right atrium, with ANP levels correlated with right atrial pressure. ANP levels are elevated in children with congenital heart disease and are further increased in children with left to right intracardiac shunts. ANP levels are increased in children with fluid overload, elevated heart rates, and critical illness. Removal of fluid from volume-overloaded patients decreases ANP and pacing-induced tachycardia increases ANP.

In order to learn more about the physiologic significance of ANP, we have studied the location of receptors for both ANP and angiotensin II (AII) in the central nervous system and in several peripheral tissues in a high-renin hypertensive rat model. We found specific ANP binding on both stellate and celiac ganglia, in the subfornical organ, choroid plexus, area postrema and pituitary of the brain; pelvis, medulla and outer cortex of the kidney in 2 kidney, 1 clip hypertensive rats. This was of great interest because these are all structures which have been said to be abnormal in hypertensive animals. We have also found specific AII binding on the anterior pituitary, glomerulosa medulla of the adrenal gland, paraventricular nucleus and subfornical organ in the brain, and in the celiac ganglia. We found a small increase in the number of AII receptors in the glomeruli in hypertensive rats. Thus, ANP and AII binding sites are present in the central nervous system of the rat and there are substantial differences between spontaneously hypertensive rats and their Wistar-Kyoto normotensive controls.

III. Renin-angiotensin system. Angiotensin II (AII) stimulates aldosterone biosynthesis and release from zona glomerulosa cells in all mammalian species studied so far. In addition, AII stimulates cortisol biosynthesis by fasciculata cells in certain mammalian species. In human adrenal glands, we found that the highest concentration of AII binding was localized to the zona glomerulosa and the adrenal medulla. Nonspecific binding was <10% of total binding. AII binding was undetectable in the rest of the adrenal cortex. Specific binding for ACE, the enzyme that converts inactive AI to active AII was highly localized in the adrenal medulla, with lower binding in the zona glomerulosa. No binding was detected in the rest of the adrenal cortex. In human pheochromocytoma, AII binding sites were undetected. However, they could be found in the zona glomerulosa in fragments of histiologically normal adrenal cortex attached to the pathological specimens. The number of ACE binding sites in human pheochromocytoma was





higher than that present in normal human adrenal. However, the differences were not statistically significant. ACE binding sites were homogeneously distributed throughout the tumor. In the rat, both AII and ACE binding sites were highly localized in the adrenal zona glomerulosa and the adrenal medulla and none was detectable in the rest of the adrenal cortex. In the bovine adrenal gland, AII binding sites were localized mostly in the zona glomerulosa and in much smaller concentrations in the rest of the cortex, but they were undetectable in the adrenal medulla. No ACE binding sites were detectable in either the bovine medulla or in any layer of the bovine cortex. Our data indicate that circulating AII could regulate aldosterone production and release in man as it does in lower animals. They also indicate that circulating AII may have a role in the release of catecholamines from adrenal chromaffin cells. The presence of a large number of ACE binding sites in human pheochromocytoma indicates that the tumor may have local production of AII. The fact that human pheochromocytomas have lost AII binding sites would further indicate that regulation of the renin-angiotensin system in human chromaffin tissues is markedly altered, perhaps due to the marked increase in catecholamine synthesis and release.

IV. Receptor regulation and ion channel modulation. Work in our laboratory and in those of others has demonstrated that binding sites of dihydropyridine, a potent  $\text{Ca}^{2+}$  channel antagonist, were up-regulated in sympathetically denervated heart, and in brain tissue of morphine-tolerant mice or after alcohol withdrawal. These findings suggest that there may be an endogenous ligand which modulates calcium channel activity and that the binding sites of dihydropyridines may be recognition sites for these endogenous calcium channel modulators.

Neurochemical studies performed in rat caudate nucleus and in hippocampus indicate that dihydropyridine-sensitive voltage-dependent calcium channels are mainly localized on intrinsic neurons and are only sparingly present or absent on nerve endings emanating from other brain areas. In addition, 3H-nitrendipine binds preferentially to a subpopulation of synaptosomes enriched in postsynaptic membrane fractions purified by Percoll gradients. An endogenous ligand has been isolated from rat brain that decreases the specific binding of 3H-nitrendipine, inhibits  $45\text{Ca}^{2+}$  uptake mediated by voltage sensitive or glutamine-receptor operated  $\text{Ca}^{2+}$  channels, decreases veratridine-elicited c-GMP formation, and reverses the enhancement of 3H-Phorbol-12, 13-dibutyrate binding after stimulation by veratridine, glutamate or kainate. Measurement of displacing activity of 3H-nitrendipine binding was used to monitor the presence of an endogenous ligand in aqueous extracts of heart or brain tissue from rats. Brain extracts contain a low molecular weight material (2000 D) that displaces 3H nitrendipine in a noncompetitive fashion, decreases voltage-dependent and glutamate receptor-dependent  $45\text{Ca}^{2+}$  uptake in cerebellar granule cells, and decreases voltage-dependent  $\text{Ca}^{2+}$  channel activity in patch clamp cardiac cells. Further research is underway to establish the structure of this substance and its physiologic function.

The chromaffin cells of canine adrenal medulla served as a model to study the regulation of the cholinergic nicotinic receptor. The role of gamma-aminobutyric acid-A (GABA-A) receptors in the regulation of the release of catecholamines and met-enkephalin-like peptides was studied by autoperfusion of the adrenal gland. Infusion into the adrenal gland of GABA-A receptor agonists (GABA, muscimol or THIP) increased the release of catecholamines and met-enkephalin peptides into the adrenal effluent blood. This release was



attenuated by the GABA-A receptor antagonist, bicucullin, but not by hexamethonium, naloxone or prior splanchnic nerve transection. In contrast, the release of catecholamines and met-enkephalin peptides elicited by splanchnic nerve stimulation was decreased by GABA-A receptor agonists and facilitated by GABA-A receptor antagonists. These data suggest that the release of these neurotransmitters may be triggered by direct stimulation of GABA-A receptors, presumably causing membrane depolarization by a burst of chloride channel opening. This type of depolarization may be responsible for obtunding the subsequent depolarizing effect of nicotinic receptor stimulation.

BHT-920 is a drug that has been shown to be effective therapeutically in the treatment of schizophrenic symptoms. We have shown by means of a number of studies of presynaptic dopaminergic processes that BHT-920 selectively modifies dopaminergic transmission in corpus striatum by reducing tyrosine hydroxylase activity. This effect was not demonstrable in other brain areas or in peripheral nervous tissue. The decrease in enzyme activity elicited by BHT-920 was attenuated by pretreatment with haloperidol. In addition, BHT-920 reduced the specific binding of spiroperidol, but not of N-propylnorapomorphine, suggesting an interaction of this compound at the D2-dopamine receptor site. These studies show that BHT-920, which is highly effective in the treatment of schizophrenic symptoms, selectively slows down the rate limiting step of dopamine synthesis, and gives credence to the view that schizophrenic symptoms may be expressed by impaired dopaminergic transmission.

Catecholamine release is generally thought to be by an exocytotic process. However, a calcium-dependent secretion of NE can be evoked in adrenergic nerves in rat heart ventricle slices incubated in a modified Krebs bicarbonate medium that contains choline chloride instead of sodium chloride. Added ATP inhibits secretion, suggesting that the nucleotide stimulates a rapid uptake of NE into vesicles proximate to the axolemma. Lithium ion, a known inhibitor of NE uptake dependent upon magnesium ATPase activity in vesicles, prevented the response to ATP. Other known inhibitors of uptake into these isolated vesicles also inhibited the response to ATP in axoplasmic vesicles, i.e., potassium ionophores, a carrier blocker, and a pH neutralizing agent for vesicles. The inhibitors increased the rate of depletion of stored NE and its deamination in non-secreting nerve endings incubated in the same medium. Valinomycin stimulated uptake in the presence of ATP. Thus mechanisms of uptake of NE in isolated vesicles also apply to the axoplasmic vesicles as well. The activity of  $Mg^{++}$  - ATPase drives protein transport to establish the electrochemical gradients of  $H^+$ , which drives the transport of NE. Isolated adrenomedullary vesicles in the presence of ATP and chloride translocate a sufficient number of ions to establish a pH and eventually cause osmotic lysis of the vesicle. By contrast, exocytosis in axoplasmic vesicles was not apparent biochemically in this system.

V. Substance P. Substance P (SP) is a neurotransmitter in both the central and peripheral nervous systems. We have shown that the SP receptor is localized to the zona inserta and lateral hypothalamus in the mid-brain of rats. The density of the receptor is reduced by 80% and 32% in these two brain areas respectively in SHR vs. normotensive control rats. We have succeeded in isolating and purifying SP receptor protein from the olfactory bulb and intestine and have shown differences in the isoelectric points and in binding displacement with SP antibodies from these two sources. Further studies are underway to explain these differences in SP receptor types. SP



and SP receptors were found densely and uniformly distributed in the uterus of rats. The receptors appeared to migrate during pregnancy towards the basal endometrium. The specific binding density of SP receptors decreased as pregnancy progressed. This disproportional growth in the uterus and its SP receptors may prove beneficial in decreasing pain perception during pregnancy.

VI. Methionine and dietary protein. We have shown previously that a low protein diet in rats causes a greater degree of hypertension and a higher incidence of stroke. This effect is reduced by increasing the amount of protein in the diet and/or by supplementation of the diet with methionine. To determine the mechanism of these effects, we sought to study the responses of aortic strips from SHRSP rats fed various protein diets. Thus aortic strips from animals fed a standard or low protein diet exhibited significantly reduced contractile responses to both NE and potassium (K<sup>+</sup>), while strips from animals fed methionine or high-protein supplemented diets demonstrated contractile responses close to those of normotensive controls. Removal of calcium from the medium caused NE-induced contractions to decrease at a faster rate in strips from animals fed standard or low-protein diets, while high-protein or methionine diets slowed the rate of decline of contractions. The contractile responses of all aortic strips to either NE or K<sup>+</sup> demonstrated a biphasic configuration consisting of a fast and a slow component. The values of both the fast and slow phases of contraction in response to NE were significantly lower in strips from standard, low-protein, and high-protein fed animals than in strips from normal or methionine-supplemented hypertensive animals. Forskolin, but not isoproterenol, produced similar relaxation in both hypertensive and normotensive aortic strips that were precontracted with NE. These studies show that variations in contractile responses are related to the protein content of the animals' diet. These contractile responses are related to the amount of intracellular free calcium and this is determined by the movement of calcium across either the cellular membrane or membranes of intracellular storage sites. These studies suggest a possible molecular defect in the pathogenesis of hypertension.

VII. Intracellular free calcium. We have measured basal levels of free intracellular calcium in platelets before and after standardized stimulation with ADP. The ADP-induced increase in intracellular calcium is blocked by prostaglandins I<sub>2</sub>, D<sub>2</sub>, E<sub>1</sub>, and E<sub>2</sub>, but not by F<sub>2</sub>α. The increase in cytosolic calcium is also blocked by dbcAMP, a synthetic analog of cAMP, which is known to be increased by prostaglandins and forskolin, which blocks the metabolism of cAMP. The increase is partially dependent on extracellular calcium, but cannot be blocked by calcium channel antagonists.

We have developed a method to grow vascular smooth muscle cells (VSMC) in monolayers on cover slips and to measure the intracellular levels of free calcium with a fluorescent dye, FURA2, in this state. Preliminary studies have shown basal calcium levels in vascular smooth muscle cells in the range of 110-130 nM. AII receptors are expressed in VSMC cultures that have been passed up to 25 times over a period of 6 months, while the response to bradykinin is markedly reduced after several passages. Bradykinin, which is a vasodilator in whole animal preparations, increases intracellular calcium levels to the same extent as AII in our test system. We are now studying the response to other hormones in this system.

Infusion of acetylcholine (ACh) into a renal artery of the dog is known



to produce an increase in renal plasma flow and sodium excretion. How ACh produces renal vasodilation and natriuresis is not clear. Recently we have found that synthesis of prostaglandins is needed to sustain the vasodilatory and natriuretic effects of ACh. We therefore studied the mechanism of renal vasoconstriction induced by ACh in indomethacin-treated dogs receiving an infusion of NDGA, a potent lipooxygenase inhibitor, or diltiazem, a calcium entry blocker. Renal arterial infusion of NDGA did not prevent the renal vasoconstriction by ACh. Renal arterial infusion of diltiazem attenuated but did not eliminate entirely the fall in renal plasma flow and sodium excretion induced by ACh in indomethacin-treated dogs. Renin secretory rate, however, did not show a rise. The data suggest that the renal vasoconstriction produced by ACh in indomethacin-treated dogs does not result from an increase in lipooxygenase activity, and furthermore, that ACh causes an increase in intracellular calcium concentration by stimulating calcium influx and the release of calcium from intracellular storage sites. The increase in cytosolic  $Ca^{+2}$  concentration then leads to contraction of vascular smooth muscle, resulting in vasoconstriction. This model should provide more useful information about the vascular responses that occur in both normal and hypertensive subjects.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01992-02 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Renal vasoconstriction induced by acetylcholine in indomethacin-treated dogs.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: John Yun Adjunct Scientist HE NHLBI

Others: John Gill Senior Investigator HE NHLBI  
Harry Keiser Chief HE NHLBI

## COOPERATING UNITS (if any)

None.

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mechanism for the renal vasoconstriction induced by acetylcholine (ACh) in indomethacin (Indo)-treated dogs was examined in dogs receiving an infusion of nordihydroguaiaretic acid (NDGA), a lipooxygenase inhibitor, or diltiazem (D), a calcium entry blocker. Renal arterial infusion of NDGA (6 mg/min) did not prevent the renal vasoconstriction by ACh. Renal arterial infusion of D (60 µg/min) attenuated, but did not eliminate entirely, the fall in renal plasma flow and sodium excretion induced by ACh (40 µg/min) in Indo-treated dogs (5 mg/kg). Renin secretory rate, however, did not show a rise. The data suggest that the renal vasoconstriction by ACh in Indo-treated dogs does not result from an increase in lipooxygenase activity. The data suggest further that ACh causes an increase in intracellular  $Ca^{++}$  concentration by stimulating  $Ca^{++}$  influx and the release of  $Ca^{++}$  from intracellular storage sites in Indo-treated dogs. The increase in cytosolic  $Ca^{++}$  concentration then leads to contraction of vascular smooth muscle resulting in renal vasoconstriction.

443



Project Description:Objective:

Infusion of acetylcholine (ACh) into a renal artery of the dog is known to produce an increase in renal plasma flow (RPF) and sodium excretion ( $\text{UNa}^{\text{V}}$ ). How ACh produces renal vasodilation and natriuresis is not clear. Recently we have found that synthesis of prostaglandins is needed to sustain the vasodilatory and natriuretic effects of ACh. In that study renal arterial infusion of ACh in control dogs produced a sustained increase in RPF and  $\text{UNa}^{\text{V}}$  without a change in glomerular filtration rate (GFR) or renin secretion rate (RSR). In dogs pretreated with indomethacin (Indo), an inhibitor of PG synthetase, renal arterial infusion of ACh produced an initial increase and then a decline in RPF and  $\text{UNa}^{\text{V}}$  that was accompanied by a progressive fall in GFR and a progressive rise in RSR. The present series of experiments was to determine whether renal arterial infusion of a calcium entry blocker, diltiazem (D) or nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, would prevent the renal vasoconstriction by ACh in Indo-treated dogs.

Methods:

Mongrel dogs were studied according to the methods reported in last year's report (Z01 HL 01992-01 HE). Three groups of dogs were studied, all were pretreated with Indo (5 mg/kg. IV bolus). One group received NDGA (6 mg/min) into one renal artery and then ACh (40  $\mu\text{g}/\text{min}$ ) was added. The second group received an infusion of D (60  $\mu\text{g}/\text{min}$ ) into one renal artery and then ACh (40  $\mu\text{g}/\text{min}$ ) was added. The third group received only D.

Major Findings and Significance:

Renal arterial infusion of NDGA in Indo-treated dogs produced no significant change in RPF ( $67 \pm 4$  ml/min  $\S$   $64 \pm 5$  ml/min). Subsequent infusion of ACh produced a rise in RPF to  $137 \pm 5$  ml/min ( $p < 0.01$ ) which was not sustained. By 100-min infusion of ACh, RPF had fallen to  $33 \pm 3$  ml/min ( $p < 0.01$ ). Thus, NDGA did not prevent the renal vasoconstriction by ACh in Indo-treated dogs.

Infusion of ACh into the renal artery of Indo-treated dogs receiving an intrarenal infusion of D produced an ipsilateral increase in  $\text{UNa}^{\text{V}}$  and RPF. The rise in  $\text{UNa}^{\text{V}}$  and RPF by ACh was not sustained at peak values. Although  $\text{UNa}^{\text{V}}$  at 100-min infusion of ACh ( $170.5 \pm 26.4$   $\mu\text{Eq}/\text{min}$ ) was still significantly higher than that before ACh infusion ( $108.5 \pm 8.4$   $\mu\text{Eq}/\text{min}$ ) ( $P < 0.05$ ), it was significantly less than that at 40-min ( $258.6 \pm 13.4$   $\mu\text{Eq}/\text{min}$ ) ( $P < 0.05$ ). RPF which was  $122.3 \pm 6.2$  ml/min before ACh infusion, increased to  $190.2 \pm 13.4$  ml/min ( $P < 0.01$ ) at 20-min infusion of ACh, but by 100-min, it had fallen to  $125.1 \pm 14.2$  ml/min ( $P < 0.01$ ). Renin secretion rate remained unchanged with the infusion of ACh. MABP was  $152 \pm 6$  mmHg during control, was unchanged at 60 min with the infusion of D ( $154 \pm 5$  mmHg) and was  $155 \pm 8$  mmHg and  $159 \pm 9$  mmHg at 20-min and 100-min infusion of ACh, respectively.



The present data suggest that the renal vasoconstriction produced by ACh in Indo-treated dogs does not result from an increase in the activity of lipooxygenase. The present findings suggest further that ACh causes an increase in intracellular  $\text{Ca}^{++}$  concentration by stimulating  $\text{Ca}^{++}$  influx and the release of  $\text{Ca}^{++}$  from intracellular storage sites in Indo-treated dogs. The increase in cytosolic  $\text{Ca}^{++}$  concentration then leads to contraction of vascular smooth muscle resulting in renal vasoconstriction.

Proposed Course of Study:

The mechanism (s) by which ACh produces renal vasoconstriction in Indo-treated dogs is unclear. If ACh produces renal vasoconstriction by an increase in cytosolic  $\text{Ca}^{++}$  concentration, then we should be able to detect this rise in intracellular  $\text{Ca}^{++}$  concentration in cell preparations with Quin-2 fluorescent indicator technique. This is currently being examined in human platelet preparations.

Publication:

None.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01996-02 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Modification of ANF and AII receptors in hypertension.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Carmen Gonzalez Visiting Fellow HE NHLBI

Others: Harry R. Keiser Chief HE NHLBI  
Juan Saavedra Unit Chief CS NIMH

## COOPERATING UNITS (if any)

None.

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.5

## PROFESSIONAL:

.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have been studying the presence of receptors for atrial natriuretic factor (ANF) and Angiotensin II (AII) in the central nervous system (CNS), and in several peripheral tissues in a high renin hypertensive rat model (2 kidney, 1 clip).

We found specific ANF and AII binding on several areas of the brain that are associated with circulatory control and we are working to determine if there is any difference in this binding between control and hypertensive animals.

We have found an increase in the number of ANF binding sites in several circumventricular areas and in the choroid plexus. These results are similar to our earlier findings in spontaneously hypertensive rats.

In peripheral organs, we have found a difference in the number of binding sites for Angiotensin converting enzyme (ACE) in renal tubules.





Project Description:Objectives:

Angiotensin is known to play an important role in body water homeostasis. Radioligand binding studies showed the presence of AII binding sites in rat brain circumventricular organs, which have been identified as the major angiotensin sensitive sites within the central nervous system. Recently, physiological antagonists of the angiotensin-renin system have been identified. These compounds are peptides produced in the mammalian cardiac atrium. One of them is ANP, which like AII has a high affinity to structures of the circumventricular organs of the rat brain.

Alterations in central AII activity occur during the pathogenesis of spontaneous hypertension. AII levels have been shown to be increased in the brain of SHR, especially in areas known to affect cardiovascular function. In addition, high affinity binding sites for AII have been found in some areas involved in cardiovascular regulation. AII binding affinity was greater in the nucleus and tract of solitarius of SHR compared to WKY with no change in the maximum binding capacity.

The objectives of the present project were: 1.) to determine if there are differences in the binding characteristics (affinity and number of receptors) as well as in the distribution of receptors for ANF and AII in control and high renin hypertensive rat models with special attention to the AV3V region in the brain. 2) To determine if there are any differences in binding characteristics in different peripheral tissues such as adrenal gland and kidney between control and high renin hypertensive rat models.

Methods:

We placed a clamp on one renal artery of Sprague Dawley rats to make a high renin hypertensive rat model. Rats were killed by decapitation. Tissues were removed and frozen by immersion in isopentane at  $-30^{\circ}\text{C}$ . Frozen tissues were cut in a cryostat at  $-14^{\circ}\text{C}$  in 16  $\mu\text{m}$  thick sections, mounted on glass slides and desiccated under vacuum at  $4^{\circ}\text{C}$  until incubation.

For AII, incubation was performed with  $^{125}\text{I}$ -Sar<sup>1</sup> AII for 60 min at room temperature in 10 nM sodium phosphate buffer pH = 7.4 containing NaCl 120 nM Na<sub>2</sub>EDTA 5 nM, Bacitracin 0.1 nM and BSA 0.2%.

For ANF, incubation was performed for 60 min at room temperature in 50 nM Tris Buffer pH = 7.4 containing NaCl 100 nM, MgCl<sub>2</sub> 5nM, Bacitracin 40 ng/ml, Leupeptin 4  $\mu\text{g}/\text{ml}$ , Chymostatin 2  $\mu\text{g}/\text{ml}$ , PMSF 0.5 ng/ml and BSD 0.5%.

\*After incubation, tissues were placed in cassettes against [<sup>3</sup>H] Ultrofilm (LKB) at room temperature for 1-3 days depending on the different concentrations used. Films were developed at  $4^{\circ}\text{C}$  for 4 min. with D19 Kodak developer.



Data analysis: A complete set of  $^{125}\text{I}$ -Standards was processed with every cassette and developed with every film. Optical densities (OD) from the ultrofilm standards and tissues were quantitated by computerized microdensitometry. After standard curve determination, OD from the tissues were interpolated from the straight line to obtain the corresponding dpm bound to the tissue. Results were corrected for the decay factor. Scatchard calculations were performed by the LIGAND computer program.

\*For ACE inhibitor binding, incubation was performed for 60 min. at room temperature in 50mM Tris-Buffer pH 7.4 containing 100mM NaCl.

#### Results and Their Significance:

In the 2 kidney, 1 clip hypertensive rats we found 1) specific  $^{125}\text{I}$  ANF binding on both, stellate and celiac ganglia, subfornical organ, choroid plexus, area postrema and pituitary of the brain; pelvis, medulla and outer cortex in the kidney. 2. Specific angiotensin II binding on the anterior pituitary, glomerulosa and medulla of the adrenal gland, paraventricular nucleus and subfornical organ in the brain and in the celiac ganglia.

Preliminary results for ANF binding, show an increase in B max in subfornical organ (SFO) and in choroid plexus in 2KC hypertensive rats when compared to normotensive controls.

We also found an increase in the B max for ACE inhibitor binding in renal tubules. The increase was about 5 times on the clipped side and about 50% on the contralateral side.

For AII we found a small increase in the number of receptors localized in the glomeruli in the hypertensive rats.

Technical problems (optical density scanning computer failure) have made it impossible to confirm these results.

Thus, ANF and AII binding sites are present in rat CNS, and we have described substantial differences between SHR and WKY rats.

If we find the same results in 2K, 1C rats as previously reported for SHR, we could hypothesize that these changes are related to the hypertension itself, rather than to the etiology of the hypertension. If the results in the two forms of hypertension are different, then they should be related to the different etiologies of the hypertension.

#### Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03520-07 HE

PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dopamine receptor regulation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Ingeborg Hanbauer

Pharmacologist

HE NHLBI

Others: Enrico Sanna  
Donald Gehlert

Visiting Fellow  
PRAT Fellow

HE NHLBI  
NINCDS

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

.4

PROFESSIONAL:

.4

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The compound BHT-920 has been shown to be therapeutically effective in the treatment of schizophrenic symptoms. The present studies showed that BHT-920 selectively modified dopaminergic transmission in corpus striatum by reducing tyrosine hydroxylase activity. This effect was not demonstrable in other brain areas or in peripheral nervous tissue. The decrease in enzyme activity elicited by BHT-920 was attenuated by haliperidol. BHT-920 reduced the specific binding of 3H-Haloperidol but not of 3H-N-propyl-norapomorphine, suggesting an interaction of this compound at the D2-dopamine receptor sites.

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Objectives:

Biochemical studies of human postmortem brain tissue indicated a hyperactive dopaminergic system. This notion was further supported by findings showing an increase in the dopamine content and in the number of D<sub>2</sub>-dopamine recognition sites in nuclei accumbens, caudatus and putamen. Our previous work was in support of this observation and showed that the coupling efficiency of GPT-binding protein was markedly increased in dopamine-rich brain areas of schizophrenics. Further evidence in line with this working model was obtained by studies of pre- and post-synaptic processes linked to dopaminergic transmission. Abundant evidence indicates that dopamine receptors exist on nigrostriatal dopaminergic terminals as well as on striatal neurons. Moreover, the presynaptic dopamine receptor appears to be very similar to the D<sub>2</sub>-subtype described on postsynaptic membranes, while the postsynaptic dopamine receptor consists mainly of D<sub>1</sub>-subtype and is positively linked to the adenylate cyclase system. The compound BHT920 was shown to act on specific presynaptic dopaminergic functions and was found to be therapeutically very effective when given to schizophrenic subjects. The present project is aimed at investigating the site of action of BHT920 and other structural analogs, and to determine the site of forskolin-sensitive adenylate cyclase system in rat brain.

Methods:

Studies on pre-synaptic dopaminergic processes include measurements of: (1) <sup>3</sup>H-dopamine uptake into striatal slices; (2) Tyrosine hydroxylase activity in various brain areas and adrenal medulla in absence and presence of p-butyrolactone; (3) L-Dopa and dopamine content in corpus striatum by HPLC technique; (4) Basal and dopamine-sensitive adenylate cyclase activity; (5) Specific binding of <sup>3</sup>H-spiroperidol, <sup>3</sup>H-n-propylnorapomorphine and <sup>3</sup>H-cocaine; and, (6) Distribution of specific binding sites of <sup>3</sup>H-forskolin in tissue sections by autoradiography.

Major Findings:

BHT920 modulates dopaminergic transmission by selectively regulating tyrosine hydroxylase activity. While BHT920 had no effect on tyrosine hydroxylase activity in vitro, it significantly reduced, in a dose-dependent manner, the striatal enzyme activity after subcutaneous injection into rats (1 mg/kg). This effect was not demonstrable in other brain areas (cerebral cortex, cerebellum, superior cervical ganglion, or in adrenal medulla). The decrease of striatal tyrosine hydroxylase was attenuated by pretreatment of rats with haloperidol (1mg/kg,i.p). Moreover, BHT-920 reduced the specific binding of <sup>3</sup>H-spiroperidol but not N-propylnorapomorphine. These results suggest that BHT-920 may specifically interact with the D<sub>2</sub>-dopamine receptor. In addition, the Na<sup>+</sup>-sensitive binding of <sup>3</sup>H-cocaine was not altered by BHT-920, indicating that the dopamine transporter system was directly affected by this compound. Studies in corpus striatum showed that forskolin-sensitive adenylate cyclase was exclusively located in intrinsic neurons of caudate nuclei and in substantia nigra in nerve terminals emanating from caudate nucleus.





Significance to Biomedical Research:

Studies on the regulation of pre- and postsynaptic dopamine receptors are imminent to improve our present knowledge on mental illness that can be attributed to impaired dopaminergic transmission (schizophrenia, tardive dyskinesia, Parkinson's disease). The outcome of the present research project showing that BHT920, which is highly effective in the treatment of the schizophrenic symptoms in selectively slowing down the rate-limiting step of dopamine synthesis, gives credence to the view that the schizophrenic symptoms may be expressed by impaired dopaminergic transmission.

Publication:

Hanbauer, I. and Sanna, E.: Molecular mechanisms involved in the desensitization of dopamine receptors in slices of corpus striatum. Progress in Brain Research, 69: 161-168, 1986.

Jennewein, H. M., Bruckwick, E. A., Hanbauer, I., Mierau, J. and Lovenberg, W.: Evidence for a specific effect of BHT-920, an azepine derivative, on tyrosine hydroxylase in corpus striatum of rats. Europ. J. Pharmacol., 123: 363-369, 1986.

Gehlert, D. R., Dawson, T. M., Filloux, F. M., Sanna, E., Hanbauer, I. and Wamsley, J. K.: Evidence that <sup>3</sup>H-forskolin binding in the substantia nigra is intrinsic to a striatalnigral projection: An autoradiographic study of rat brain. Neuroscience Lett. 73, 114-118, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03552-03 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulatory mechanisms for voltage-dependent Ca<sup>2+</sup> channels in rat brain

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Enrico Sanna Visiting Fellow HE NHLBI

Others: Ingeborg Hanbauer Pharmacologist HE NHLBI  
Gilbert Wright, Jr. Research Chemist HE NHLBI

## COOPERATING UNITS (if any)

Merrell Dow Research Institute, Cincinnati, OH (Phillip Robinson)

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

From neurochemical lesion studies performed in rat caudate nucleus and in hippocampus, it appears that dihydropyridine-sensitive voltage-dependent calcium channels are mainly localized on intrinsic neurons and are only sparingly present or absent on nerve endings emanating from other brain areas.

3H-Nitrendipine binds preferentially to a subpopulation of synaptosomes enriched in postsynaptic membrane fractions purified by Percoll gradient.

Endogenous ligand (EL) for 3H-nitrendipine binding sites has been isolated from rat brain:

EL decreases the specific binding of 3H-nitrendipine; it inhibits 45Ca<sup>2+</sup> uptake mediated by voltage-sensitive or glutamate receptor-operated Ca<sup>2+</sup> channels; it decreases the veratridine-elicited c-GMP formation and reverses the enhancement of 3H-Phorbol-12, 13-dibutyrate binding after stimulation by veratridine, glutamate or kainate.



Project Description:Objective:

It is now well accepted that calcium channel antagonists and agonists interact with specific recognition sites, which appear to constitute a molecular component of the supramolecular unit of the voltage-dependent calcium channel. In neurons, it has been suggested by electrophysiological and pharmacological studies that there is a coexistence of multiple types of voltage-dependent calcium channels, and that the action of dihydropyridine-like compounds occurs at the "L-type" or "long-lasting current" calcium channel. It has also been shown that the dihydropyridine-sensitive voltage-dependent calcium channels are not involved in presynaptic processes, so that dihydropyridine-like compound are generally unable to regulate biochemical mechanisms such as the release of neurotransmitters. A physiological role for the dihydropyridine recognition sites in the regulation of the calcium channel activity, then, has not yet been elucidated. However, recent reports in the literature support the hypothesis that these recognition sites may be important as binding sites for an endogenous regulator of calcium channel activity. Recent work in this laboratory has demonstrated the existence in rat brain of an endogenous substance that displaces the  $^3\text{H}$ -nitrendipine binding from its recognition sites and that decreases the calcium channel function.

Methods:

Synaptosomal fractions were isolated from rat striatum by Percoll gradient centrifugation according to Robinson and Lovenberg (1986). Aliquots of striatal homogenate ( $S_1$ ) were loaded over a Percoll discontinuous density gradient consisting of 23%, 15%, 10% and 3% Percoll, and after centrifugation, the interfacial fractions were collected and used for the biochemical studies.

$^3\text{H}$ -Nitrendipine binding is routinely used to monitor the presence of the endogenous ligand and to assess the amount of activity contained in a given fraction during the purification steps.  $^3\text{H}$ -Nitrendipine binding is measured by incubating hippo-campal membrane suspension aliquots (150-200 g protein) in the presence of 25-400 pM  $^3\text{H}$ -nitrendipine. Primary cultures of cerebellar granule cells were prepared as described by Gallo, et al. (1982). Cerebella from 7-day old rats were trypsinized and the cells were resuspended in culture medium and aliquots of  $2.5$  to  $3.0 \times 10^6$  cells were added to each culture dish. Eight to ten-day old primary cultures were used for measuring the  $^{45}\text{Ca}^{2+}$  uptake and c-GMP formation in presence and absence of veratridine, glutamic acid and kainic acid.

$^3\text{H}$ -Phorbol-12,13-dibutyrate binding is measured by incubation of membrane suspension prepared from cerebellar granule cells in culture in presence of 5-100 nM  $^3\text{H}$ -PDB.



Evidence for a specific localization of voltage-dependent  $\text{Ca}^{2+}$  channels in cell bodies of caudate nucleus:

Intrastriatal injection of kainic acid caused the destruction of a large percentage of interneurons in the caudate nucleus and abolished about 90% of the specific binding for  $^3\text{H}$ -nitrendipine. In addition, the basal and the veratridine-stimulated  $^{45}\text{Ca}^{2+}$  uptake measured in slices of kainic acid-lesioned caudate nucleus were greatly reduced. In contrast, infusion of 6-OH-DA or 5,7-DHT into the lateral ventricle failed to alter the density of  $^3\text{H}$ -nitrendipine binding sites in caudate nucleus.

Two subpopulations of synaptosomes were purified by a discontinuous Percoll gradient. The values of multiple biochemical parameters suggest that one subpopulation (called fr. 4) contains mainly presynaptic terminals, while the other one (fr. 3) is enriched in postsynaptic membrane fractions. In agreement with the data on the localization,  $^3\text{H}$ -nitrendipine binding sites are present with an higher density in fr. 3 than in fr. 4.

Modulatory function of an endogenous ligand extracted from rat brain on  $\text{Ca}^{2+}$  channel activity:

Recently, in our laboratory, an endogenous substance that has an inhibitory action on  $\text{Ca}^{2+}$  channel activity has been isolated and purified from rat brain extracts. Scatchard analysis of the  $^3\text{H}$ -nitrendipine binding to hippocampal membranes indicates that the density of the recognition sites is reduced, in a non-competitive manner, in the presence of the endogenous ligand from 250 to 84 fmol/mg prot. In addition, the veratridine-stimulated  $^{45}\text{Ca}^{2+}$  uptake and c-GMP formation were inhibited in a dose-dependent and reversible manner. Furthermore, EL inhibited the glutamate-elicited  $^{45}\text{Ca}^{2+}$  uptake in cultured granule cells, but EL was not able to affect the binding of  $^3\text{H}$ -glutamic acid to hippocampal membranes. To further support the previous data on the inhibitory action of EL on the  $\text{Ca}^{2+}$  channels, we have preliminarily studied the effect of EL on the  $^3\text{H}$ -phorbol-12,13-dibutyrate ( $^3\text{H}$ -PDB) binding to membrane suspension prepared from cultured granule cells. Incubation of the cells in presence of veratridine, glutamic acid or kainic acid increased from 30 to 90% the density of  $^3\text{H}$ -PDB recognition sites. However, this stimulatory effect was reduced when the cells were preincubated in presence of EL.

Publications:

- 1) Sanna, E., Head, G.A., and Hanbauer, I.: Evidence for a selective localization of voltage-sensitive  $\text{Ca}^{2+}$  channels in nerve cell bodies of corpus striatum. J. Neurochem. 47: 1552-1557, 1986.
- 2) Hanbauer, I. and Sanna, E.: Presence in brain of an endogenous ligand for nitrendipine binding sites that modulates  $\text{Ca}^{2+}$  channel activity. A.N.Y.A.S., 1987.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03553-02 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Anterior pituitary-atrial regulation: A novel endocrine axis.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Nadav Zamir Visiting Fellow HE NHLBI

Others: Peter Ohman Visiting Associate HE NHLBI  
Harry Keiser Chief HE NHLBI

## COOPERATING UNITS (if any)

None.

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Mammalian atrial myocytes contain biologically active peptides within specific secretory granules. These peptides, collectively termed atrial natriuretic peptides (ANP), have potent natriuretic, diuretic and vascular smooth muscle relaxant activities and thus are of potential importance in controlling blood pressure. Little is known about the regulation of ANP secretion into the blood stream. Atrial distension by increased perfusion pressure causes release of ANP in the rat heart-lung preparation, and acute volume expansion in rats also causes a marked increase in circulating ANP. Administration of pharmacological doses of arginine-Vasopressin and oxytocin induced a profound release of ANP into the circulation. The stimulated release of ANP apparently was related to increased arterial blood pressure and could be mimicked by bolus injection of the pressor agents angiotensin II and phenylephrine. In a series of experiments, we examined the role of the pituitary gland in basal and stimulated (acute volume expansion) release of ANP.

505



Project Description:Methods:

Experiments were performed on male, 10-12 weeks old Sprague-Dawley rats. All animals were kept in individual cages and were under alternate 12 h periods of dark and light (light on from 06.00 to 18.00 h) in controlled temperature and humidity and were given rat chow and water ad libitum.

Rats were conscious and unrestrained in their home cages throughout the experiments. Arterial blood pressure and heart rate were continuously monitored throughout the experiment. Blood pressure and heart rate were allowed to stabilize for 20 min and then blood samples were withdrawn. The blood was replaced with an equal volume of pooled rat blood. Blood volume expansion was caused by infusion of 5% dextrose 20 ml/kg at 37°C over 1 min. via the femoral vein. Blood samples were taken 15 min after the stimulus, when peak levels occur. ANP was measured by radioimmunoassay developed and validated in this laboratory.

Results and Their Significance:

Blood volume expansion induced a rapid and profound release (2-3 fold) of ANP in conscious sham operated animals.

To investigate the significance of the pituitary gland in ANP release, basal and stimulated circulating levels of ANP were measured in hypophysectomized conscious rats 1,2,8 and 30 days after operation. Hypophysectomized rats have significantly lower mean arterial pressures compared to sham operated animals. Basal and stimulated release of ANP were significantly blunted in these animals. The concentrations of ANP in left and right atria of hypophysectomized rats (2 and 8 days) were either normal or higher than that of the sham operated animals. These results excluded the possibility that depleted atrial ANP stores in hypophysectomized rats are responsible for the reduced ANP release.

Although a hemodynamic factor remains a possibility for the reduced ANP release in hypophysectomized rats, additional data implicating the involvement of the pituitary gland came from experiments on hypophysectomized rats bearing ectopic pituitary autotransplants for 10 days. Pituitary grafted animals have low mean arterial pressures similar to that of hypophysectomized rats. Right atrial pressures of hypophysectomized animals with or without pituitary grafts were similar under basal conditions and following acute volume expansion. However, basal and stimulated release of ANP were completely restored in the pituitary grafted animals.



To test which lobe of the pituitary gland has a role in ANP release, we have used hypophysectomized rats bearing either anterior lobe or neurointermediate lobe of pituitary gland transplanted to the kidney. Sham hypophysectomized animals served as controls for both groups. Experiments were performed 10 days after surgery. Acute volume expansion caused marked increases in ANP release in both sham hypophysectomized rats (+260%) and hypophysectomized rats bearing anterior pituitary autotransplant (+149%). No significant effect on ANP release in response to acute volume expansion was observed in hypophysectomized rats bearing a neurointermediate lobe autotransplant.

Ectopic pituitary transplants are known to produce large amounts of prolactin and little, if any, LH, FSH or GH. Thus, it was reasonable to suspect that the effects of the transplants on circulating ANP levels observed in the present study might be due to prolactin. This was examined by using intravenous administration of prolactin (2  $\mu$ g per animal). This had no effect on basal or stimulated release of ANP 0.5-2 hrs later. Furthermore, bolus administration of pharmacological doses of drugs which modify circulating levels of prolactin, such as bromocriptine or haloperidol for 2-4 hrs had no effect on basal or stimulated release of ANP. However, long term physiological effects of prolactin will also be tested in order to better elucidate the role of prolactin in ANP release.

The normal ANP secretion in pituitary grafted animals raises several intriguing possibilities. It could be that hormones other than prolactin, GH, LH, and FSH secreted from the anterior pituitary gland act directly on the atrial myocyte to facilitate the physiological stimuli induced release of ANP. The action could be receptor mediated or through membranous changes. Alternatively, hormones derived from the pituitary gland could affect ANP release indirectly through the action on other endocrine organs such as the thyroid or adrenal cortex. The existence of an "atriotrophic factor" should also be considered. More work is needed to distinguish between these possibilities and to test them.

In conclusion we suggest that pituitary gland-atria interactions play a major role in the regulation of fluid and electrolyte homeostasis via ANP secretion. In future experiments, we will try to isolate and characterize the "atriotrophic factor" and test its physiological role.



Publication:

- 1) Zamir, N., Skofitsch, G., Eskay, R., and Jacobowitz, D.M. Distribution of immunoreactive atrial natriuretic peptide in the central nervous system of the rat. Brain Res. 365, 105-111 (1986).
- 2) Eskay, R.L., Zukowska-Grojec, Z., Haass, M., Dave, J.R. and Zamir, N. Circulating atrial natriuretic peptides in conscious rats: Regulation of release by multiple factors. Science 232, 636-639 (1986).
- 3) Eiden, L.E. and Zamir, N. Metorphamide levels in chromaffin cells increase after treatment with reserpine. J. Neurochem. 47, 1651-1654, (1986).
- 4) Zamir, N., Skofitsch, G., and Jacobowitz, D.M. Distribution of immunoreactive-melanin concentrating hormone in the central nervous system of the rat. Brain Res. 373, 240-245 (1986).
- 5) Haass, M., Zamir, N., and Zukowska-Grojec, Z. Plasma levels of atrial natriuretic peptides in conscious adult spontaneously hypertensive rats. Clin. Exp. Hyper. A8, 277-288 (1986).
- 6) Zamir, N., and Maixner, W. The relationship between cardiovascular and pain regulatory systems. Ann.N.Acad. Sci. 467, 371-384 (1986).
- 7) Zukowska-Grojec, Z., Haass, M., Kopin, I., and Zamir, N. The interactions of atrial natriuretic peptides with the sympathetic and endocrine system in the pithed rat. J. Pharmacol. Exp. Ther. 239(2), 480-487 (1986).
- 8) Haass, M., Zukowska-Grojec, Z., Kopin, I., and Zamir, N. Role of autonomic nervous system and vasoactive hormones in the release of atrial natriuretic peptides in conscious rats. J. Cardiovasc. Pharmacol. (in press).
- 9) Zamir, N., Haass, M., Dave, J.R. and Zukowska-Grojec, Z. Anterior pituitary gland modulates the release of atrial natriuretic peptides from cardiac atria. Proc Natl Acad. Sci. USA 84, 541-545 (1987).





## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03555-02 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of atriopeptin III on the baroreflex.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E. Marks	Adjunct Scientist	HE NHLBI
Others:	P. Ohman	Visiting Associate	HE NHLBI
	H. Keiser	Chief	HE NHLBI

## COOPERATING UNITS (if any)

None.

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mammalian atrial tissue contains a peptide referred to as atrial natriuretic factor (ANF) that can be released under a variety of conditions into the general circulation. We have shown previously that atriopeptin III (APIII), a synthetic form of ANF, decreases cardiac output and lowers mean arterial pressure. Lack of reflex cardioacceleration to the APIII-induced reduction in blood pressure prompted an investigation into the effect of APIII on baroreceptor function in the intact conscious rat.

Mean arterial pressure changes were elicited with bolus injections of phenylephrine (PE) and nitroprusside (NP). The dose of APIII employed did not change basal heart rate, mean arterial pressure (MAP) or the MAP response to PE or NP. The bradycardiac response to PE was accentuated and the baroreflex sensitivity increased by APIII. No significant effect on NP-induced changes in heart rate were observed.

In the conscious rat, APIII selectively enhances the baroreflex response to increases, but not decreases, in arterial pressure.

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Objectives:

Experiments described in annual report Z01 HL 03555-01 HE were extended to further clarify the effect of APIII on heart rate and baroreceptor function. It has been suggested that ANF alters cardiac output through stimulation of cardiopulmonary vagal afferents that in turn would diminish sympathetic outflow. This effect could manifest itself as an alteration in heart rate and baroreflex responsivity. We studied the effect of APIII on the heart to pharmacologic induced alterations in blood pressure.

Methods:

Sprague-Dawley rats were anesthetized prior to bilateral femoral vein and right femoral artery catheterization for drug infusion and measurement and recording of blood pressure and heart rate. Following a minimum of 18 hours recovery, each rat received bolus injections of phenylephrine (0.5-4.0  $\mu\text{g/kg}$  iv) and nitroprusside (1.0-4.0  $\mu\text{g/kg}$  iv). Arterial pressure and heart rate returned to baseline between injections. Thirty minutes after the last bolus, a continuous infusion of APIII (0.1  $\mu\text{g/kg/min}$ ) was begun and the phenylephrine and nitroprusside doses were repeated.

Results:

The dose of APIII did not affect basal blood pressure, heart rate, or the magnitude of the blood pressure response to either PE or NP. The maximal change in heart period (HP) (msec) was determined during the initial ten beats of the corresponding maximal change in mean arterial pressure (MAP). Baroreflex sensitivity was defined by the  $\Delta\text{HP}/\Delta\text{MAP}$  ratio.

The increase in heart period after PE was significantly greater during APIII infusion. Baroreflex sensitivity to PE was  $0.997 \pm 0.07$  msec/mmHg during APIII vs  $0.613 \pm 0.08$  during vehicle ( $p < 0.005$ ). APIII did not alter the onset of the maximal heart rate response following the maximal change in MAP ( $1.29 \pm 0.1$  sec vs  $1.2 \pm 0.15$  sec). APIII infusion did not affect the tachycardiac response to NP administration. Baroreflex sensitivity to NP was unchanged at  $1.45 \pm 0.3$  msec/mmHg before vs  $1.43 \pm 0.2$  during APIII infusion.

Significance:

The role of atrial natriuretic factor in volume homeostasis and its potential therapeutic value in the treatment of hypertension and disorders involving fluid overload continues to be of major interest. Experiments performed in the intact conscious animal undergoing a variety of physiologic and pathophysiologic changes provides fundamental information concerning the mechanism of action of this peptide.

Proposed Course of the Study:

The baroreceptor studies have been completed. An abstract has been submitted to the American Heart Association scientific sessions and a manuscript is in preparation. The effect of APIII on vagal afferents and renal function is presently under study.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03556-01 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Angiotensin II and converting enzyme binding in adrenal gland and pheochromocytomas

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Gonzalez-Garcia Visiting Fellow HE NHLBI

Others: H.R. Keiser Chief HE NHLBI  
J.M. Saavedra Chief, Unit of Preclinical Pharm., Sect. on Clin. Pharm. LCS NIMH

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.5

## PROFESSIONAL:

.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Binding sites for Angiotensin II (AII) and Angiotensin Converting Enzyme (ACE) were studied in human adrenal glands and pheochromocytomas and in rat and bovine adrenals. In human and rat adrenals, both AII and ACE binding sites were localized in the medulla and zona glomerulosa, being absent in the rest of the cortex. In bovine adrenals, ACE binding sites were not detectable. The number of AII binding sites was higher in the zona glomerulosa, lower in the rest of the cortex and not detectable in the medulla.

In human pheochromocytomas, ACE binding sites were homogeneously distributed throughout the tumor. However, AII binding sites could not be detected.

These results suggest that circulating and/or locally formed AII could regulate the adrenomedullary and zona glomerulosa functions in man. Our findings also indicate that chromaffin cell tumors retain their ACE binding sites while losing their AII binding sites. These results indicate alterations in the regulation of the renin-angiotensin system in chromaffin tumors.



Project Description:Objectives:

Angiotensin II (ANG II) has direct actions on mammalian adrenal glands. In the adrenal cortex, ANG II stimulates aldosterone biosynthesis and release from the zona glomerulosa cells in all mammalian species studied (Douglas et al, 1978; Catt et al, 1979). In addition, ANG II stimulates cortisol biosynthesis by fasciculata cells, an action which is species-dependent (Carpenter et al, 1961; Kaplan et al, 1962).

In the adrenal medulla, ANG II has been proposed to play a modulatory role in the release of catecholamines from chromaffin cells (Feldberg and Lewis, 1964; Peach, 1971; Reit, 1972; Zimmerman, 1978; Ungar and Phillips, 1983).

Binding sites for ANG II have been described in the rat adrenal cortex zona glomerulosa with membrane binding techniques (Douglas et al, 1984) and in both rat zona glomerulosa and adrenal medulla with autoradiographic methods (Israel et al, 1985a; Healy et al, 1985). Binding to angiotensin converting enzyme (ACE, Kininase II, E.C. 3.4.15.1) was also detected by autoradiography, with the use of a  $^{125}\text{I}$ -labeled specific ACE inhibitor in the rat adrenal medulla (Plunkett et al, 1985).

These results, the presence of renin activity in human adrenal glands (Naruse et al, 1983) and the existence of renin, ANG II and ACE activity in neuroblastoma and glioma cell lines (Fishman et al, 1981; Okamura et al, 1984), suggest the possibility of an active ANG II system in mammalian adrenal medulla. In addition, human pheochromocytomas contain ACE (Narse et al, 1983; Mizuno et al, 1985b) indicating that such a system could also be active in chromaffin cell tumors. We asked the question whether the ANG II system was present in human adrenal gland and in pheochromocytoma. We utilized autoradiographic methods to localize and quantify ANG II and ACE binding in discrete areas of the human adrenal glands and in human pheochromocytomas obtained at surgery and we compared the localization of the human binding to that present in rat and bovine adrenals.

Methods:

Rat, bovine and human tissues (normal adrenal glands and pheochromocytomas) were cut in small pieces and frozen in isopentane at  $-30^{\circ}\text{C}$  and stored at  $-70^{\circ}\text{C}$  until used.

Frozen rat, cow and human tissues were cut in 16  $\mu\text{m}$  thick sections in a cryostat at  $-19^{\circ}\text{C}$ , mounted on gelation-coated slides and desiccated under vacuum at  $4^{\circ}\text{C}$  for 1 to 5 days until incubation.

For ANG II binding sites, tissues were preincubated for 15 min. at room temperature in 10 mM sodium phosphate buffer pH 7.4, containing NaCl (120 mM),  $\text{Na}_2\text{EDTA}$  (5 mM), bacitracin (0.1 mM), and bovine serum albumin (0.2%), and then incubated for 60 min in fresh buffer with 0.6 mM of  $^{125}\text{I}$ -ANG II. Nonspecific binding was determined in the presence of 5  $\mu\text{M}$  unlabeled ANG II (Israel et al., 1984). After incubation, the slides were washed four times, 1 min. each, with ice-cold 50mM Tris-HCl buffer, pH 7.56 and dried under a cold stream of air.





ACE was localized and enzyme levels were quantified by incubating tissue sections with 0.2 nM of the ACE inhibitor [ $^{125}\text{I}$ ]-351A in fresh Tris-HCl buffer, pH 7.4 containing 100 mM NaCl for 60 min at room temperature (Correa et al, 1985). Non specific binding was determined in adjacent sections in the presence of 2 uM unlabelled 351A. All sections were preincubated for 30 min in the incubation buffer in the presence (non specific binding ) or absence (total binding ) of 0.1 uM cold 351A (Corea et al, 1985). After incubation, all sections were washed 4 times, 60 s each, in ice-cold 50 mM Tris-HCl, pH 7.4, and dried under a cold stream of air.

For autoradiography, sections were placed in X-ray cassettes (CGR Medical Corp. Baltimore, MD.) and placed in immediate contact with [ $^3\text{H}$ ] Ultrofilm (LKB Industries, Rockville, MD) for 4-7 days depending on the concentration of binding sites. Optimum exposure time for each tissue and binding site studied wre determined in preliminary experiments. A complete set of standards was processed with every cassette.

### Results and Their Significance:

#### ANG II and ACE in human adrenal glands

In human adrenal glands the highest concentration ANG II binding was localized to the zona glomerulosa and the adrenal medulla. Non specific binding was less than 10% of the total binding. ANG II binding was not detectable in the rest of the adrenal cortex.

Specific ACE binding sites were highly localized in the adrenal medulla, with lower binding in the zona glomerulosa. No binding was detected in the rest of the cortex.

#### ANG II and ACE in human pheochromocytomas

ANG II binding sites could not be detected in human pheochromocytomas. However, they could be found in the zona glomerulosa when fragments of histologically normal adrenal cortex were present in the pathology specimens.

The number of ACE binding sites in human pheochromocytoma was higher than that present in normal human adrenal. However, the differences were not statistically significant. ACE binding sites were homogeneously distributed through the tumor.

#### ANG II and ACE binding in rat and bovine adrenals

In the rat, both ANG II and ACE binding sites were highly localized in the adrenal zona glomerulosa and adrenal medulla, and were not detectable in the rest of the cortex.

In the bovine adrenal gland, ANG II binding sites were localized mostly in the zona glomerulosa, and in much smaller concentrations in the rest of the cortex, but they were not detectable in the adrenal medulla. No ACE binding sites were detectable in either the bovine medulla or in any layer of the bovine cortex.



Significance:

Our finding of a high number of ANG II binding sites in human adrenal gland, supports the hypothesis that circulating ANG II could regulate aldosterone production and release in humans as is the case in lower mammals.

ANG II binding sites were present in the human adrenal medulla, indicating the possibility of a role for circulating ANG in the metabolism of chromaffin cells and probably in catecholamine release.

The presence of high ACE binding sites in human pheochromocytomas indicates the possibility of local ANG II formation in these tumors.

Human pheochromocytomas had a large number of ACE binding sites but they had lost ANG II binding sites. These results indicate that regulation of the renin-angiotensin system is altered in human chromaffin tumors perhaps due to the marked increases in catecholamine synthesis and release typical of these tumors.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  ZOI HL 03557-01 HE
PERIOD COVERED Oct. 1, 1986 to Sept. 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Effect of sodium intake on renal DOPA metabolism.		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  PI: John R. Gill Senior Investigator HE NHLBI  Other: David Goldstein Senior Investigator HE NHLBI		
COOPERATING UNITS (if any)  None.		
LAB/BRANCH Hypertension-Endocrine Branch		
SECTION		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)  In a previous study, protein feeding, which normally increases urinary dopamine without a change in plasma DOPA, was shown to increase plasma DOPA when DOPA decarboxylase was inhibited. These observations led to the conclusion that protein feeding increased the delivery of DOPA to the kidney, thereby increasing renal formation of dopamine. Since an increase in sodium intake also stimulates renal formation of dopamine, the possibility that it does so through changes in DOPA metabolism was studied. When sodium intake was decreased from 109 to 9 mEq/day in normal subjects on a constant metabolic diet, renal clearance and excretion of DOPA decreased. When sodium intake was increased from 9 to 249 mEq/d, renal clearance and excretion of DOPA increased by 82% and 62% respectively; the excretion of dopamine increased by 23%. The changes in renal clearance of DOPA occurred without a change in plasma DOPA. The results are consistent with the interpretation that changes in sodium intake may affect renal dopamine formation by altering DOPA metabolism and, in turn, its delivery to the kidney.		

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Project Description:

Considerable evidence indicates that urinary dopamine increases when sodium intake is increased acutely by infusion of normal saline or chronically by oral supplementation. The increase in sodium excretion parallels the increase in urinary dopamine and may, at least in part, be caused by it. In vitro studies of segments of proximal tubule indicate that dopamine inhibits  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  activity in these cells, an action that may increase sodium excretion by decreasing proximal tubular reabsorption. The effect of dopamine on proximal tubule segments is also produced by l-DOPA, a precursor, that is converted to dopamine by DOPA decarboxylase in those cells. Recent studies indicate that protein feeding increases circulating DOPA and this is thought to be the basis for the increase in renal dopamine generation and sodium excretion that also occur. The present studies were designed to determine if changes in dopamine formation that accompany changes in sodium intake are also associated with changes in DOPA metabolism.

Methods:

Six normal subjects were admitted to an air-conditioned ward and were fed a constant metabolic diet containing 109 mEq/d of sodium (normal sodium) for seven days, 9 mEq/d of sodium (low sodium) for seven days and then 249 mEq/d of sodium (high sodium) for seven days. Plasma for assay of DOPA was collected on the last day of each of the three sodium intakes. All urine was collected throughout the study and analyzed for sodium, DOPA and dopamine.

Results:

When sodium intake was decreased from normal to low mean, DOPA and dopamine excretion decreased from 13.9 to 10 and 133  $\mu\text{g}/\text{day}$ , respectively. When sodium intake was then increased from low to high, mean DOPA and dopamine excretion increased from 10 and 133  $\mu\text{g}/\text{day}$  to 16.2 and 164  $\mu\text{g}/\text{day}$ , respectively. The changes in mean DOPA excretion were associated with similar directional changes in the mean clearance of DOPA per 100 ml. glomerular filtrate. Mean plasma DOPA was similar for all three sodium intakes. The results are summarized in the following table.

Sodium Intake mEq/d	Plasma DOPA Pg/ml	Urinary DOPA $\mu\text{g}/\text{d}$	Clearance DOPA ml/100 ml GFR	Urinary Dopamine $\mu\text{g}/\text{d}$
109	2442 $\pm 486$	13.9 $\pm 2.2$	6.0 $\pm 2.0$	150 $\pm 34$
9	2466 $\pm 476$	10.0 $\pm 2.1$	3.9 $\pm 1.0$	133 $\pm 24$
249	2431 $\pm 561$	16.2 $\pm 3.4$	7.1 $\pm 2.2$	164 $\pm 34$

values = Mean  $\pm$  S.E.

N = 6

:





The considerable changes in renal clearance and excretion of DOPA produced by changes in sodium intake without an apparent change in plasma DOPA concentration are consistent with the notion that changes in sodium intake may have led to changes in the amount of DOPA presented to the kidney. The differences in clearance of DOPA during the three sodium intakes and, presumably, in the amount of DOPA available to DOPA decarboxylase in the renal tubule may explain the differences in dopamine formation. Thus, these preliminary studies are consistent with the hypothesis that sodium intake, like protein intake, may regulate renal dopamine formation and, in turn, sodium excretion via regulation of DOPA metabolism.

#### Proposed Course of Study:

Although urinary dopamine may vary over a wide range and be influenced by a number of factors such as the intakes of protein and sodium, the mechanism by which the body regulates renal dopamine, whether by neuronal release or extraneuronal formation, has not been clear. The present studies support the growing body of evidence that DOPA delivery to the kidney for extraneuronal dopamine formation may constitute an important regulatory mechanism. Future studies in normal subjects will be designed to obtain more direct evidence that sodium loading increases the entry of DOPA into the circulation. If the findings indicate that increased entry of DOPA does occur, studies to identify the source will be performed.

Studies in patients with idiopathic hypertension indicate that urinary dopamine is significantly higher in salt-resistant patients given a high sodium intake than it is in salt-sensitive patients. Future studies will be performed to determine if DOPA metabolism is different and could be the basis for the difference in renal dopamine generation in these two hypertensive subsets.

#### Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 HL 03558-01 HE
PERIOD COVERED Oct. 1, 1986 to Sept. 30, 1987		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Dietary protein and defect in vascular smooth muscle contractility in hypertension.		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Martina Diolulu	Guest Researcher	HE NHLBI
Others: Tom Ropchak	Biologist	HE NHLBI
COOPERATING UNITS (if any) None		
LAB/BRANCH Hypertension-Endocrine Branch		
SECTION		
INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Thoracic aortas were isolated from 4-month-old, stroke-prone spontaneously hypertensive rats (SHRSP) and age-matched normotensive Wistar Kyotos (WKY) which had been maintained for 3 months on one of four experimental diets: standard (STD) (24% protein), low protein (LP) (19% protein), high protein (HP) (32% protein), or high methionine (Met) (1.9% methionine). The isometric tensions produced by low concentrations of norepinephrine (10-10, 10-9M) were similar in aortic strips from both SHRSP under various experimental diets (SHRSP/diet) and WKY under the standard diet (WKY/STD). The strips from SHRSP/STD and SHRSP/LP exhibited significantly reduced contractile responses to high doses of NE (10-8, 10-7M) and K+ (60 mM) while strips from SHRSP/Met and SHRSP/HP demonstrated contractile response values close to normal. Removal of Ca from the medium caused NE-induced contractions to decrease at a faster rate in strips from SHRSP/STD than in WKY/STD strips. The presence of LP in SHRSP strips accelerated the rate while Met and HP slowed the rate of decline of contraction. The contractile responses of all experimental strips to either NE or K+ demonstrated a biphasic configuration consisting of a fast and a slow component. The values of both the fast and slow components in response to NE were significantly lower in strips from SHRSP/STD, SHRSP/LP and SHRSP/HP than in strips from WKY/STD and SHRSP/Met. Forskolin produced similar relaxation in strips from both SHRSP/diets and WKY/STD that were precontracted with NE. The results from these experiments indicate that (1) the differences in contractile response of aortic strips from normotensive WKY and SHRSP may be attributed essentially to the biochemical make-up of the membrane vesicles involved in Ca2+ transport and (2) in contrast to aortae from SHRSP/Met and SHRSP/HP, the aortae from SHRSP/STD and SHRSP/LP seem to have a much lower ability to retain and thus release appropriate amounts of Ca2+ required for muscle contraction.		



Project Description:Objectives:

An increase in total peripheral resistance is generally accepted as the major factor in the maintenance of high arterial pressure in both man and experimental animals. The hypotheses for the increase in vascular resistance include an increase in vascular tone (a state of maintained contraction) and/or alteration in the reactivity of the vascular smooth muscle cells.

One finding common to several laboratory studies is that the maximum force of contraction generated by strips of isolated vascular smooth muscle from hypertensive animals is less than that of comparable strips from normotensive animals.

Calcium ion at physiological levels is essential for the coupling of membrane excitation with tension development by the contractile protein. Numerous studies have demonstrated reduced ability of the sarcoplasmic reticulum (SR) and other  $\text{Ca}^{2+}$ -trap vesicles from hypertensive rats to take up and store  $\text{Ca}^{2+}$ . Such a defect in uptake as well as a consequent inappropriate release of  $\text{Ca}^{2+}$  could alter the physiology of excitation--contractile coupling.

Since a high protein diet modifies the  $\text{Ca}^{2+}$ -transport activities of the SR from spontaneously hypertensive rats, this study examines the effect of long-term feeding of dietary protein on the responsiveness of isolated aortic strips from stroke-prone spontaneously hypertensive rats to stimulating and relaxing agents.

Methods:

Forty male spontaneously hypertensive rats (SHR/A3N) 4 weeks of age were divided into four equal groups of 10 rats and each group was randomly assigned to one of the four experimental diets; Low protein (LP) (19% protein), standard (STD) (24% protein), high protein (HP) (32% protein), or high methionine (1.9% methionine) (MET) for 3-months. For reactivity studies, the thoracic aorta was removed cut into helical strips, and the responses of the strip to drugs were then recorded isometrically.

The Parameters Determined were:

1. Contractile responses of all experimental aortic strips to norepinephrine ( $\text{NE } 10^{-10}$ - $10^{-7}\text{M}$ ) and potassium ( $\text{K}^+$ , 60 mM).
2. The rate of reduction of NE-induced contraction when aortic strips were incubated in calcium free medium.
3. Relaxation responses of aortic strips to B-adrenoreceptor or adenylate cyclase stimulant.



Major Findings:

When compared to WKY the aortic strips from SHRSP demonstrated reduced contractile force development in response to norepinephrine and potassium. Differences were observed in contractile responses among strips from SHRSP under various experimental diets (SHRSP/diets). The strips from SHRSP/STD and SHRSP/LP exhibited significantly reduced contractile responses to both norepinephrine and potassium, while strips from SHRSP/Met and SHRSP/HP demonstrated contractile response values close to normal. Removal of Ca from the medium caused norepinephrine-induced contractions to decrease at a faster rate in strips from SHRSP/STD than in WKY/STD strips. The presence of low protein (LP) in SHRSP strips accelerated the rate while high protein (HP) and methionine supplement (Met) slowed the rate of decline of contraction. The contractile responses of all aortic strips to either norepinephrine or potassium demonstrated a biphasic configuration consisting of a fast and a slow component. The values of both the fast and slow phases of contraction in response to norepinephrine were significantly lower in strips from SHRSP/STD, as well as SHRSP/LP, and SHRSP/HP than in strips from WKY/STD and SHRSP/Met. However, the values of the fast but not the slow phases of contraction in response to norepinephrine were similar in strips from SHRSP/Met and WKY/STD. Forskolin, but not isoproterenol, produced similar relaxation in both hypertensive and normotensive aortic strips that were precontracted with norepinephrine.

Significance of Biomedical Research:

In essential hypertension, increased peripheral resistance and elevated arterial pressure seem causally related to an increase in vascular smooth muscle tone. As in the case of skeletal and cardiac muscle, the amount of calcium present at the contractile apparatus appears to regulate tone, or the contractile state of smooth muscle. The two possible mechanisms that could regulate the availability of calcium ion in the cytoplasm of the smooth muscle cell include the movement of calcium across plasma membranes and the sequestration and release of calcium by intracellular storage sites. Abnormalities in each of these mechanisms have been implicated in the pathogenesis of hypertension. The finding that dietary protein as specific amino acids may lower blood pressure, provides a possible clue to the biochemical basis for hypertension. Continued research on the molecular mechanisms of the protective effect of dietary protein may suggest useful preventive measures in hypertension.





## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03559-01 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987.

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Catechols and sympathoadrenomedullary function in health and disease.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: David Goldstein, Senior Investigator, HE, NHLBI.

Others: R. Stull, Chemist, HE, NHLBI; G. Eisenhofer, Visiting Fellow, NINCDS; B. Chidakel, Electronics Technician, BEIB DRS; C.J. Folio, Nurse, HE NHLBI; F.L. Sax, MSF, CB, NHLBI; J. Brush, MSF, CB, NHLBI; J. Bacher, Chief, VRB, DRS; R. Udelsman, MSF, DEB, NICHD; T. Ropchak, Biologist, HE, NHLBI; R. Zimlichman, Visiting Associate, HE, NHLBI; G. Kelly, Biologist, HE, NHLBI; I.J. Kopin, Scientific Director, OD, NINCDS; H.R. Keiser, Chief, HE, NHLBI.

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## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We developed and applied new techniques for assessing sympathoadrenomedullary function at rest, during stress, and in disease states by using levels of the sympathetic neurotransmitter norepinephrine (NE), the hormone epinephrine (E), the catecholamine precursor dihydroxyphenylalanine (DOPA), and the norepinephrine metabolite dihydroxyphenylglycol (DHPG) to indicate various aspects of neurotransmitter synthesis, release, re-uptake, and metabolism. A pharmacokinetic approach including systemic intravenous infusions of tracer-labelled NE and isoproterenol was used to discover that changes in total body spillover of NE into arterial blood provided better agreement with sympathetically-mediated cardiovascular responses during mental challenge than did measurements of changes in antecubital venous or arterial NE. The heart was found to be particularly dependent on neuronal uptake for removal of NE. Techniques were developed to provide in vivo estimates of NE levels at post-synaptic adrenoceptors and at neuronal uptake sites. Plasma DHPG was found to indicate intraneuronal disposition of axoplasmic NE. DOPA in the bloodstream was found to emanate from sympathetic nerves and to provide a major source of dopamine (DA) production in the kidney. Changes in plasma DOPA appeared to indicate changes in the rate of catecholamine biosynthesis. Sympathoneural and adrenomedullary activity were found to be regulated differentially during various stresses. Positron emission tomography is being studied as a possible means to visualize sympathetic function. A large number of collaborative studies about catechols are summarized.



### *Background and Objectives*

Virtually every activity of our lives is associated with changes in the activity of the sympathoadrenomedullary system, changes which usually are silent but which are vital for preserving the constancy of the internal environment. A large number of physical or psychiatric disorders, or medications to treat them, influence sympathetic function. The challenge for several years has not been the discovery of this system or its importance, especially in the elaboration of stress responses, but its role in the pathophysiology of disease. And the challenge about understanding its pathophysiologic role has been the development of valid techniques to assess its activity.

Norepinephrine is the sympathetic neurotransmitter, and after the introduction of assay techniques for measuring levels of norepinephrine in the bloodstream, evidence rapidly accumulated that plasma norepinephrine was related to activity of the sympathetic nervous system. In humans, we found, for instance, that orthostatic hypotension due to peripheral sympathetic degeneration was associated with low levels of plasma norepinephrine; and that patients with regional sympathectomies did not have the usual arteriovenous increment of norepinephrine.

The catecholamine biosynthetic cascade is thought to begin with uptake of tyrosine into sympathetic nerve endings (See Figure). The specific enzymatic hydroxylation of tyrosine to DOPA is the rate-limiting step in catecholamine biosynthesis. DA is taken up into vesicles containing dopamine-beta-hydroxylase, or DBH, and converted to norepinephrine. DBH, like tyrosine hydroxylase, occurs only in tissues which synthesize norepinephrine, but unlike tyrosine hydroxylase DBH occurs only in the vesicles. Products of tyrosine hydroxylase, DOPA, dopamine, and norepinephrine, can feedback-inhibit tyrosine hydroxylase activity. Reuptake of released norepinephrine is the predominant means to terminate its actions. This energy-requiring, non-stereoselective process is called Uptake-1. It can be blocked by desipramine or cocaine.

Norepinephrine in the axonal cytoplasm can be taken up into vesicles by a stereoselective process or can be oxidized by mitochondrial monamine oxidase (MAO). The deaminated product is an aldehyde which is rapidly converted to dihydroxyphenylglycol (DHPG). The uptake of norepinephrine into storage vesicles can be inhibited by reserpine. Extraneuronal tissues remove norepinephrine by a different process, which is inhibited by metanephrine and hydrocortisone. Extraneuronal tissues contain catechol-O-methyltransferase (COMT), which converts norepinephrine to normetanephrine. COMT exists primarily in extraneuronal tissues, especially in the liver and kidney. Products of both MAO and COMT activity include vanillylmandelic acid (VMA) and methoxyhydroxyphenylglycol (MHPG).

Norepinephrine can regulate its own release by stimulating pre-synaptic, inhibitory alpha-2 adrenoceptors. A large variety of other biochemicals can modulate norepinephrine release, but for simplicity these aren't shown. Yohimbine blocks alpha-2 receptors and increases norepinephrine release, whereas clonidine stimulates those receptors and decreases norepinephrine release.

(A) The use of antecubital venous levels of NE to indicate sympathoneural activity has been



controversial. Antecubital venous levels of norepinephrine are determined importantly by local release and can fail to detect changes in sympathetic activity elsewhere. The relationship between sympathetic activity and plasma norepinephrine is determined to an important extent by neuronal uptake, as discussed below. Norepinephrine removal is blood flow-dependent.

We wished to determine the relationships between changes in antecubital venous NE and cardiovascular responses to stress which are known to be mediated mainly by the sympathetic nervous system. We compared the strength of these relationships with those obtained using arterial NE or using total body spillover of NE into arterial blood, based on a pharmacokinetic approach including systemic intravenous infusions of tracer-labelled NE.

In patients undergoing cardiac catheterization, we conducted similar infusions of tracer-labelled NE and isoproterenol to determine for the first time the in vivo rate of release, removal, and neuronal uptake of NE in the human heart.

(B) We determined the source and meaning of plasma levels of DHPG. Since neural tissue lacks catechol-O-methyltransferase, we hypothesized that plasma DHPG can indicate intraneuronal metabolism of cytoplasmic NE. We could then apply this knowledge to estimate NE concentrations at neuronal uptake sites, according to concepts presented in the Methods section below.

(C) As noted previously, DOPA is the precursor of all the catecholamines and is the immediate product of the rate-limiting step in catecholamine biosynthesis, namely, hydroxylation of tyrosine. We found that substantial amounts of endogenous DOPA circulate in the bloodstream. The plasma concentration is about 10 times that of norepinephrine. Where does this DOPA come from, and what does it mean?

One's first reaction to this question might be that plasma DOPA derives from the diet. DOPA is an amino acid, and trace amounts of it probably are ingested all the time. Several lines of evidence we have obtained suggest that this cannot be the only explanation for plasma DOPA. Instead, plasma DOPA may emanate from sympathetic nerve endings. If so, then changes in plasma DOPA may indicate changes in catecholamine biosynthesis, since the enzymatic conversion of tyrosine to DOPA is thought to be the rate-limiting step in the catecholamine biosynthetic cascade. Also, if plasma DOPA were found to emanate from neural tissue, then it may function as an indirectly-acting neurohormone, allowing synthesis of DA even in tissues devoid of tyrosine hydroxylase.

(D) A new and potentially important approach in the assessment of sympathetic function would be the development of a technique to visualize sites of synthesis and release of NE. We have embarked on a project designed to do this, based on systemic intravenous injections of positron-emitting 2-fluoro-DA.

These considerations led us to develop and apply new and more refined techniques for assessing sympathetic function: (A) the measurement of total body and regional spillover of norepinephrine into the bloodstream and neuronal uptake of NE; (B) the use of plasma DHPG to indicate intraneuronal disposition of norepinephrine, with estimations of NE levels at post-synaptic adrenoceptors and Uptake-1 sites; (C) the use of changes in plasma DOPA to indicate changes in the rate of catecholamine biosynthesis; and (D) positron emission imaging of



the sympathetic nervous system.

(E) Finally, during the past year we collaborated in a large number of studies involving measurements of catechols in subjects undergoing stress or suffering from various cardiovascular or neurologic diseases. The findings are summarized in the section about Results and Their Significance.

### *Methods*

#### *(A) Measurement of total body and regional release of norepinephrine*

We determined whether changes in antecubital venous norepinephrine were related to the cardiovascular changes that occur during a laboratory challenge--playing a video game--which is known to be associated with increased pulse rate and blood pressure. We measured arterial as well as antecubital venous norepinephrine, and we also infused tracer-labelled norepinephrine to provide estimates of the total body rate of spillover of norepinephrine into the bloodstream (6). In patients undergoing cardiac catheterization a similar pharmacokinetic approach was applied to measure cardiac spillover, removal, and neuronal uptake of NE.

In the tracer-infusion technique, radioactive norepinephrine is infused at such a low dose that no hemodynamic effects occur at all. The clearance of norepinephrine from the bloodstream can be calculated from the infusion rate of the tracer divided by the steady-state concentration of radioactive norepinephrine in the arterial plasma. The apparent spillover rate of norepinephrine into plasma can then be calculated as the product of plasma norepinephrine and norepinephrine clearance. Analogous calculations can be made of regional clearance and spillover of norepinephrine, based on arterial and venous norepinephrine, the regional percent extraction of radioactive norepinephrine, and regional blood flow.

During infusion of tritiated I-NE, when blood samples were obtained simultaneously from the brachial artery and antecubital vein, a bit more than half of the labelled catecholamine was removed in the arm. Since normally a slight arteriovenous increment in plasma norepinephrine is observed in the arm, norepinephrine must have been released at a substantial rate in the arm. This has important implications, as explained below.

#### *(B) Use of plasma DHPG to indicate intraneuronal disposition of norepinephrine*

According to the concepts in the Figure, plasma DHPG is thought to emanate from cytoplasmic norepinephrine deaminated by monoamine oxidase. We developed liquid chromatographic-electrochemical methodology for measuring levels of DHPG in plasma, urine, tissue, and cerebrospinal fluid, and we used this methodology to obtain evidence in vitro in rat vas deferens that DHPG is a major intra-neuronal metabolite of NE and, as described below, that plasma DHPG in man derives from intraneuronal metabolism of norepinephrine.

We have found that *plasma norepinephrine can under certain circumstances be used to estimate the concentration of norepinephrine at post-synaptic adrenoceptors*. This can allow one to take into account the large and variable concentration gradient for norepinephrine between the synaptic clefts and the plasma. We applied similar reasoning to estimate NE levels at





neuronal uptake sites using changes in plasma DHPG.

The several removal processes for norepinephrine produce a concentration gradient between the synaptic cleft and the plasma. Because of these processes, during an infusion of norepinephrine to achieve a given pressor response, the steady-state norepinephrine concentration in the plasma must exceed that at vascular neuroeffector junctions. Similarly, during stimulation of release of endogenous norepinephrine to achieve the same pressor response, the steady-state norepinephrine concentration in the plasma must be less than that at vascular neuroeffector junctions. The mean cleft norepinephrine concentration associated with a given pressor response must be somewhere between the steady-state plasma concentrations measured during norepinephrine release and during norepinephrine infusion and, assuming that the gradient is similar in both directions, can be estimated from the geometric mean of the two values.

This "window" for estimating cleft norepinephrine is quite large. In man, in order to produce an increment of 20 mm Hg by infusing norepinephrine, the venous norepinephrine concentration often must exceed 2000 pg/ml, whereas during pressor responses of similar magnitude induced by release of endogenous norepinephrine, the venous norepinephrine level increases by only a few hundred pg/ml or less.

The "window" for estimated cleft norepinephrine can be made much smaller by blocking Uptake-1. This reduces the concentration gradient for norepinephrine between the synapse and the plasma. After blockade of Uptake-1 using desipramine, there was a marked shift to the left in the pressor-log plasma norepinephrine relationship during norepinephrine infusion and a symmetric shift to the right in this relationship during sympathetic stimulation in rats. The combinations of blocking reflexive circulatory controls, alpha-2 adrenoceptors, and Uptake-1, allowed us to estimate cleft norepinephrine, first in rats and then in people. Despite quite different experimental procedures, the results were about the same.

We then used plasma DHPG to estimate norepinephrine concentrations at Uptake-1 sites, according to the following reasoning:

Plasma DHPG during NE release or NE infusion should have the same source--uptake of NE by Uptake-1. The concentration gradient between the neuroeffector junctions and the plasma during NE release was assumed to be the reciprocal of the NE concentration gradient during NE infusion. For a given increment in the mean arterial level of DHPG, the change in the arterial NE concentration was about 1 log unit smaller for stimulation of NE release (by tilt or yohimbine administration) than for NE infusion, and the average change in the NE concentration at Uptake-1 sites must have been the geometric mean between the change in arterial NE concentrations for release and for infusion.

(C) Plasma levels of DOPA were measured in a large number of clinical and laboratory settings, including sympathectomy, sympatholytic treatments, ganglion blockade, inhibition of catecholamine biosynthesis, manipulations designed to alter axoplasmic NE, neuroblastoma, and phrochromocytoma. The role of circulating DOPA in urinary DA excretion was assessed in dogs by infusing tritiated DOPA into a renal artery. Effects of dietary salt loading or restriction on plasma and urinary DOPA were assessed in normal volunteers.



## Results and Their Significance

(A) During playing the video game, blood pressure, pulse rate, cardiac output, and forearm blood flow all increased. Antecubital venous levels of norepinephrine were unchanged. Arterial levels of norepinephrine increased significantly; and total body spillover of norepinephrine increased to a substantial extent. *Whereas changes in antecubital venous norepinephrine were unrelated to changes in systolic blood pressure or cardiac output during the game, changes in total body spillover of norepinephrine clearly were related to the circulatory changes.*

In the forearm, as blood flow increased, the clearance of norepinephrine increased linearly. The slope of this relationship was less than 1, however, because as the blood flow increased, the proportionate extraction of arterial norepinephrine decreased exponentially. As the cardiac output--which is the total body blood flow-- increased, the total body clearance of norepinephrine increased as well. This was because of increased delivery of norepinephrine to removal sites such as the liver and kidney when the cardiac output was increased. In a setting where cardiac output is increased, in order to maintain a given arterial level of norepinephrine, the spillover of norepinephrine into the bloodstream must be increased too. In essence, *the calculation of total body spillover of norepinephrine takes into account the blood flow-dependence of norepinephrine clearance. Changes in antecubital venous norepinephrine can underestimate regional spillover of norepinephrine when forearm blood flow is increased, and changes in arterial norepinephrine can underestimate total body spillover of norepinephrine when cardiac output is increased.* The use of the total body and regional spillover technique, therefore, appears to be a distinct improvement in the assessment of sympathetic activity and its hemodynamic consequences compared with antecubital venous levels of norepinephrine.

We have begun to apply the tracer-infusion technique in patients 40 years old or younger who have essential hypertension, in order to determine whether excessive sympathoadrenomedullary responsiveness occurs early in the development of the disorder. Preliminary results based on 12 such patients have indicated that: *^(1) Hypertensives had normal rates of spillover of NE into arterial blood at rest and normal increments in total body spillover of NE during mental challenge; and (2) Hypertensives had excessive increases in mean arterial pressure and total peripheral resistance to blood flow during mental challenge. These findings, if confirmed during the coming year, would suggest that vascular hyper-responsiveness to endogenously released norepinephrine occurs early in the development of essential hypertension.*

We also have begun to apply this technique to assess rates of *cardiac release and neuronal uptake of NE* in patients with hypertrophic cardiomyopathy and control patients with chest pain and normal coronary arteries. Preliminary results based on 11 patients and 3 controls have included a surprisingly large amount of Uptake-1 activity in the heart, based on a large arteriovenous increment in plasma DHPG (see below), a much larger proportion of NE removal in the heart than isoproterenol removal, and a large amount of radioactive DHPG production by the heart during infusion of tracer-labelled NE (see below). This is a potentially important finding, regardless of the pathophysiology of hypertrophic cardiomyopathy, because *if the heart is virtually completely dependent on Uptake-1 to remove NE, then this may explain the susceptibility of the heart to cocaine toxicity, since cocaine releases NE and inhibits Uptake-1.*

We also are applying the tracer-infusion technique to determine for the first time the



*relationship in vivo between physiologic changes in regional sympathetic nerve activity and physiologic changes in regional release of NE into the bloodstream.* This application is based on direct recording of renal post-ganglionic sympathetic nerve activity in anesthetized rats and calculations of renal NE spillover using arterial and venous concentrations of NE, renal blood flow measured using Doppler flowmetry, and regional extraction of tracer-labelled NE at baseline, during nitroprusside-induced hypotension, and during phenylephrine-induced hypertension.

(B) When alpha-2 adrenoceptors were blocked using yohimbine, then plasma norepinephrine, blood pressure, and plasma levels of DHPG were increased; but when neuronal uptake of norepinephrine was blocked using desipramine, then yohimbine no longer increased plasma DHPG, even though the norepinephrine and blood pressure responses were enhanced. Therefore the increments in plasma DHPG caused by yohimbine were due to re-uptake and intra-neuronal metabolism of released norepinephrine.

When norepinephrine was infused to produce pressor responses of 10, 20, and 30 mm of Hg, plasma DHPG increased; but in subjects pre-treated with desipramine, increases in plasma norepinephrine during norepinephrine infusion were not associated with increases in plasma DHPG.

*These findings provide the first clinical evidence that plasma DHPG derives from and can indicate intraneuronal metabolism of NE.*

*In both rats and in healthy people, a 20 mm Hg sympathetically-mediated pressor response was associated with about a 560 pg/ml (3 nM) concentration of norepinephrine in the average neuroeffector junction (or, more accurately, at post-synaptic alpha-1 receptors).*

The model for estimating junctional norepinephrine concentrations associated with pressor responses circumvents the problem of possible distortion of the junctional-plasma norepinephrine gradients by variability of norepinephrine removal mechanisms and so makes more accurate the interpretation of circulating norepinephrine levels in terms of sympathetically-mediated norepinephrine release.

*At an increment of about 100 pg/ml in arterial DHPG, the estimated increment in NE level at Uptake-1 sites was between about 500 and 600 pg/ml.* This value agreed well with values described above in humans and rats based on blood pressure responses.

After administration of clonidine to inhibit norepinephrine release, we found that plasma DHPG decreased to a plateau level of about 700 to 800 pg/ml, in contrast with plasma norepinephrine, which decreased to very low levels. Since plasma DHPG appears to derive from sympathetic nerve endings after metabolism of cytoplasmic norepinephrine, the most likely explanation for the continued production of DHPG when norepinephrine release was inhibited is leakage of norepinephrine from the storage vesicles into the cytoplasm. This leakage process can be quite active. Thus, we found that in conscious rats, administration of reserpine markedly increased plasma DHPG as well as increments in radioactive DHPG during administration of tracer-labelled tritiated NE. Indeed, *the regional rate of production of DHPG after administration of clonidine may provide a clinical index of the rate of leakage of NE from storage vesicles into the cytoplasm.*



The results lead to the following suggestions about plasma DHPG:

Increments in plasma DHPG during sympathetic stimulation reflect re-uptake and metabolism of released norepinephrine back into the axonal cytoplasm; and when release of norepinephrine is small, then plasma DHPG indicates leakage and metabolism of stored norepinephrine from the vesicles into the cytoplasm.

As part of the protocol to measure junctional norepinephrine concentrations, the alpha-2 adrenoceptor antagonist yohimbine has been administered to young patients with essential hypertension and normotensive control subjects. Preliminary findings have indicated that *about 1/2 of the hypertensive patients--but none of the controls--had marked hypertensive, sympathoadrenomedullary, and behavioral responses to yohimbine. It is possible, therefore, that in a substantial proportion of young patients with hypertension, alpha-2 adrenoceptors in the brain rein in sympathetic outflow, even during mild laboratory challenges, but when these receptors are blocked, then previously unrecognized increased sympathoadrenomedullary activity becomes apparent.*

(C) When plasma DOPA was measured in an artery and in the antecubital vein of the arm, the levels in the vein virtually always were higher. This is consistent with regional production of DOPA and not with an exclusively dietary source. We found that in patients who had undergone a regional sympathectomy, the concentration of DOPA in the arm vein was lower than in an artery, in contrast with the arteriovenous increment in plasma DOPA in the intact limbs. This provides strong clinical support for the notion that plasma DOPA can derive, at least partially, from sympathetic nerve endings. Analogously, we found that plasma DOPA levels virtually always were increased in patients with neuroblastoma and usually were increased in patients with malignant pheochromocytoma, tumors of neural crest origin.

Since DOPA is the product of hydroxylation of tyrosine, and since tyrosine hydroxylase is found exclusively in tissues which can synthesize catecholamines, we measured the effect on plasma DOPA when catecholamine synthesis was inhibited using alpha-methyl-para-tyrosine, which competitively inhibits the rate-limiting hydroxylation step. One day after a single dose of alpha-methyl-paratyrosine, we found that plasma DOPA was decreased by a bit over 60%. This suggests that *plasma DOPA derives to a substantial extent from catecholamine-synthesizing tissues.*

We also investigated factors which might affect plasma DOPA. Addition of a reducing agent to blood immediately after withdrawal of the sample resulted in more than a doubling of plasma DOPA. Tilt, which rapidly increases plasma norepinephrine, had little effect on plasma DOPA in normal volunteers, so that the two catechols probably are not co-released. General anesthesia decreased plasma DOPA by up to 75% in dogs and monkeys. In anesthetized dogs, prolonged ganglion blockade decreased plasma DOPA, and prolonged nitroprusside-induced hypotension increased plasma DOPA. The latter findings are consistent with the view that changes in plasma DOPA can reflect changes in tyrosine hydroxylase activity.

Further support for this notion was obtained in a study involving simultaneous infusion of trace amounts of tritiated NE and various physiologically active amounts of unlabelled NE. As the amount of unlabelled NE was increased, the plasma level of DOPA decreased, and inhibition of oxidative deamination of cytoplasmic NE using a monoamine oxidase inhibitor enhanced this suppressant effect on plasma DOPA. These findings suggest that *as cytoplasmic NE increases, the*





*amount of feedback inhibition of tyrosine hydroxylase increases, resulting in decreased production of DOPA and decreased plasma levels of DOPA.*

If plasma DOPA derived from catecholamine-synthesizing tissues of the body, then this would have two important implications: first, *circulating DOPA could provide a substrate for the creation of dopamine even in cells which lack tyrosine hydroxylase, such as renal tubular cells; and second, the rate of regional production of DOPA might be related to the regional rate of catecholamine biosynthesis.* Regarding the first implication, we have obtained evidence in dogs that *at least 1/2 of urinary dopamine (DA) derives not from renal dopaminergic nerves but from plasma DOPA.* In proximal tubular cells of the kidney, DOPA is converted extensively to DA, and DA, in turn, inhibits Na-K ATPase and stimulates natriuresis. We have obtained clinical data in both outpatients and inpatients demonstrating that *during a high-salt diet, the excretion of DOPA doubled* without a change in plasma DOPA. In a subject who received the DOPA decarboxylase inhibitor, carbidopa, daily, urinary DOPA excretion increased markedly by about 10-fold, and urinary DA excretion was cut in half. If confirmed in other subjects, these findings would be consistent with the view that *DOPA is a neurohormone which can inhibit Na-K ATPase indirectly; that DOPA continually is being released into the bloodstream at a high rate and actively is removed in the kidney; and that renal uptake of DOPA is enhanced during salt loading.*

(D) *We recently began to develop methodology for visualization of the sympathetic nervous system.* This methodology is based on intravenous injection of positron-emitting fluoro-DA and subsequent imaging of regional positron emission. Dopamine is known to be an excellent substrate for neuronal uptake. After its uptake into sympathetic nerve endings, DA is taken up into storage vesicles, converted to NE, and the NE slowly released. The rate of this release is an index of NE turnover. We have succeeded in synthesizing 2-fluoro-DA rapidly from 2-fluoro-DOPA and positron-emitting 2-fluoro-DA rapidly from positron-emitting 2-fluoro-DOPA. We also have conducted unilateral superior cervical ganglionectomies in foxhounds, producing unilateral Horner's syndrome. During the coming year we will inject positron-emitting 2-fluoro-DA into these dogs. After uptake of the 2-fluoro-DA into sympathetic nerve endings, a process requiring only a few minutes, we expect to find unilateral loss of positron emission in the dog's head due to the cervical ganglionectomy. Other dog, rat, and in vitro studies are planned to characterize further the uptake of 2-fluoro-DA, conversion of 2-fluoro-DA to 2-fluoro-NE, and retention and turnover of 2-fluoro-NE. Eventually we hope to be able to visualize sympathetic function in regions such as the heart in humans.

(E) During the past year, we also conducted the following projects:

1. In collaboration with Dr. Alan Davis, of the Children's Hospital, we measured *plasma levels of immunoreactive atrial natriuretic factor (irANF)* in children with congenital heart disease, tachycardia, altered blood volume, or critical illness. The results indicated that irANF in children mainly is produced by the right atrium, with ANF levels correlated with right atrial pressure; irANF levels are elevated in children with congenital heart disease; irANF levels are increased further in children with left-to-right intracardiac shunts; irANF levels are increased in children with fluid overload, elevated heart rates, and critical illness; removal of fluid from volume-overloaded patients decreases irANF; and pacing-induced tachycardia increases irANF.

2. In collaboration with Dr. Arno Zaritsky, of the Children's Hospital, we developed the *first assay technique to measure simultaneously plasma levels of dobutamine, dopamine, and*



*isoproterenol*, which are commonly used pressor agents in the treatment of clinical shock. We discovered that blood levels of these substances during steady-state infusions were poorly related to the infusion rates, because patients with hepatic or renal failure had markedly prolonged clearances of the drugs. The results reinforce the suggestion that clinical responses, not infusion rates determined by algorithm, should provide the guidelines in administering these drugs to critically ill patients.

3. In collaboration with Dr. Suzan Nadi, of NINCDS, we measured for the first time levels of catechols *in vivo* in normal human brain and in epileptogenic foci. We found that the predominant catechols in normal human cortical tissue were NE, DA, and DOPA. We also found that in epileptogenic foci, levels of all these catechols were significantly increased, consistent with a compensatory inhibitory function of catecholamines in the cerebral cortex.

4. In collaboration with Dr. Alan Breier, of NIMH, we measured the *circulatory and neuroendocrine effects of acute glucoprivic stress in humans*. Administration of 2-deoxyglucose caused plasma cortisol to triple and plasma epinephrine to increase about 40-fold. In contrast, plasma NE increased less than 2-fold. The results demonstrate that *adrenomedullary and sympathoneural responses during stress can be differentiated* and that adrenomedullary and pituitary-adrenocortical stimulation during glucoprivic stress are more closely linked than sympathoneural and pituitary-adrenocortical stimulation.

5. In collaboration with Dr. Robert Udelsman, of NICHD, we determined *circulatory and neuroendocrine effects of surgical stress in humans*. Whereas surgery was unassociated with changes in plasma catecholamines, cortisol, or ACTH, extubation and recovery were associated with large, simultaneous increases in ACTH, cortisol, and plasma epinephrine. Plasma NE was unchanged. The results provide further support for a close linkage between *adrenomedullary and pituitary-adrenocortical activation during stress, independent of sympathoneural activation*.

6. In collaboration with Dr. Julianne Imperato-McGinley, of the Cornell Medical Center, NY, we demonstrated *reversibility of catecholamine-induced dilated cardiomyopathy in pheochromocytoma*. The results indicated that *even end-stage, dilated cardiomyopathy can be reversed eventually if the underlying cause is cured*.

7. We observed a *dissociation between ACTH and catecholamine responses to isoproterenol in humans*. Whereas isoproterenol increased plasma levels of NE (19) and plasma renin activity, plasma levels of epinephrine and ACTH were unaffected. The results again indicate the *dissociation of sympathoneural from adrenomedullary activation and cast doubt on the hypothesis of Axelrod and others that circulating beta-adrenoceptor agonists can stimulate ACTH release*.

8. In collaboration with Dr. Reuven Zimlichman, now at the Soroka Medical Center, Ben-Gurion University of the Negev, Beer-Sheva, Israel, we conducted several studies about *cytoplasmic levels of ionized calcium ([Ca<sup>++</sup>]<sub>i</sub>)*. We found that platelet levels of [Ca<sup>++</sup>]<sub>i</sub> were normal in spontaneously hypertensive rats, that acute inhibition of Na-K ATPase by ouabain did not affect [Ca<sup>++</sup>]<sub>i</sub> in platelets, lymphocytes, or adrenomedullary cells, and that angiotensin II stimulated increases in [Ca<sup>++</sup>]<sub>i</sub> and catecholamine release in cultured bovine adrenomedullary cells.

9. In collaboration with Dr. Emanuel Troullos, of the NIDR, we determined *cardiovascular and neuroendocrine effects of inclusion of epinephrine in local anesthetics used in dental surgery*.



We found that one minute after injection of local anesthetic containing 1:100,000 (144 µg) epinephrine, mean plasma epinephrine was 27.5 times higher than baseline, associated with elevations in systolic pressure, heart rate, and a 33% increase in rate-pressure product. The results indicate that *significant amounts of epinephrine can be systemically absorbed following intraoral injection* and that *the absorbed epinephrine can alter the cardiovascular status of the patient*, including myocardial oxygen consumption.

10. In collaboration with Dr. Jaylan Turkkan, of The Johns Hopkins School of Medicine, we produced the *first primate model of DOCA-salt hypertension*. We found that whereas salt loading alone had little chronic effect on blood pressure in baboons, combined DOCA and salt produced marked chronic elevations in pressure. The hypertension was not associated with increased plasma levels of catecholamines and was reversed by diuretic therapy (combined hydrochlorothiazide and triamterene) but not by sympatholytic therapy (clonidine or atenolol). The results indicated that *DOCA-salt hypertension in baboons is not determined by increased sympathetic activity*, in contrast with DOCA-salt hypertension in rats but more consistent with clinical findings. *This non-human primate model of a volume-loading form of hypertension is more applicable to the clinical condition of steroid-salt hypertension than is the rat model.*

11 Clonidine suppression testing was conducted in outpatients after week-long periods of dietary salt loading or restriction. We found no effect of dietary salt intake on results of the test. *The clonidine suppression test, used in the diagnostic evaluation of pheochromocytoma, can be conducted in outpatients without concern about possible effects of inter-individual differences in dietary salt intake.*

12 In collaboration with Dr. Robert Udelsman, of NICHD, we determined circulatory and neuroendocrine responses to surgical stress in adrenalectomized, mineralocorticoid-replaced monkeys. *Whereas adrenalectomy had little effect on plasma catecholamines at rest, plasma NE responses to surgical stress were markedly enhanced in adrenalectomized monkeys, and the magnitude of this enhancement was inversely related to the amount of glucocorticoid pre-treatment.* The results indicate *compensatory sympathoneural activation during surgical stress in adrenalectomized monkeys*, with the magnitude of this activation related to the amount of glucocorticoid.

13 We measured effects of ganglion blockade on cerebrospinal fluid NE in anesthetized dogs. All studies to date about cerebrospinal fluid NE in essential hypertension have reported significantly increased cerebrospinal fluid NE in hypertensive patients. The source and meaning of cerebrospinal fluid NE have been poorly understood. We found that ganglion blockade using trimethaphan markedly decreased cerebrospinal fluid NE, even when blockade-associated hypotension was prevented by concurrent administration of phenylephrine. Ganglion blockade also abolished reflexive increases in cerebrospinal fluid NE as observed during nitroprusside-induced hypotension. The results indicated that *NE in cerebrospinal fluid may have a similar origin and meaning as NE in plasma: release of the sympathetic neurotransmitter from post-ganglionic sympathetic nerve endings.* During the coming year we will attempt to obtain further support for this hypothesis by measuring cerebrospinal NE in dogs with bilateral superior cervical ganglionectomies.

#### *Proposed Course of Project:*

(A) During the coming year we will complete our assessment of plasma norepinephrine



pharmacokinetics during mental challenge and during yohimbine administration in essential hypertension. We also will complete a validation study of the impedance cardiographic technique, upon which the above-described findings about total peripheral resistance were based, by simultaneous comparisons with cardiac output values obtained using green dye dilution. The study of human cardiac release, removal and neuronal uptake of NE will be completed.

(B) During the coming year we will complete large studies using rates of release of DHPG and NE to provide more complete assessments of sympathetic function than previously available in vasa deferentia and plasma of spontaneously hypertensive rats. Methodology will be developed to measure plasma normetanephrine. The combined measurement of DHPG and normetanephrine will allow for the first time in vivo assessments of the relative roles of intra- and extra-neuronal metabolism in the disposition of NE.

(C) During the coming year we will complete several large studies about relationships between changes in plasma DOPA and tyrosine hydroxylase activity and about plasma DOPA uptake and conversion to DA in the kidney as a determinant of natriuresis and blood volume homeostasis during volume loading.

(D) During the coming year we hope to be able to conduct the first positron emission tomographic scans of sites of NE synthesis and release.

(E) During the coming year we will be continuing to collaborate with investigators in others Institutes and medical centers in the general area of assessments of sympathoadrenomedullary function.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03560-01 HE

## PERIOD COVERED

Oct. 1, 1986 through Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Intracellular free calcium in platelets and vascular smooth muscle cells.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Peter Ohman	Visiting Associate	HE NHLBI
Others:	John Yun	Adjunct Scientist	HE NHLBI
	John Reid	Adjunct Scientist	HE NHLBI
	Margaret Hill	Med. Tech.	HE NHLBI
	Harry Keiser	Chief	HE NHLBI

## COOPERATING UNITS (if any)

None.

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.4

## PROFESSIONAL:

.4

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have measured basal levels of free intracellular calcium in platelets before and after standardized stimulation with ADP. The ADP-induced increase in intracellular calcium is blocked by prostaglandins I<sub>2</sub>, D<sub>2</sub>, E<sub>1</sub>, and E<sub>2</sub>, but not by F<sub>2</sub> α. The increase in cytosolic calcium is also blocked by dbcAMP, a synthetic analog of cAMP, which is known to be increased by prosta-glandins and forskolin, which blocks the metabolism of cAMP. The increase is partially dependent on extracellular calcium, but cannot be blocked by calcium channel antagonists.

We have developed a method to grow vascular smooth muscle cells (VSMC) in monolayers on cover slips and to measure the intracellular levels of free calcium with a fluorescent agent, Fura2, in this state. Preliminary studies have shown that certain types of receptors are lost from these cells during serial passages.

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Objectives:

- 1) To measure the basal levels of free intracellular calcium in platelets from normal humans and compare the aggregatory response with the increase in intracellular calcium induced by submaximal stimulatory doses of ADP, and to evaluate the modulation of the response induced by different prostanoids and drugs that alter their mediators.
- 2) To study the interaction of vasoactive hormones, i.e., angiotensin II, norepinephrine, epinephrine, bradykinin, and atrial natriuretic peptide on the levels of intracellular free calcium in vascular smooth muscle cells.

Methods:

The intracellular level of free calcium is measured in vitro in platelets from normal volunteers using the fluorescent calcium indicator, Quin2. Aggregation is measured in vitro with an aggregometer.

In vitro culture of VSMC from the rat in monolayer was developed in our laboratory and is now done on cover slips so that the cells can be studied during the earliest passages. Measurements of intracellular levels of free calcium are performed with the calcium fluorophor Fura2.

Results and Their Significance:

The level of free intracellular calcium in platelets from normal humans is 40-60 nM. The increase induced by a submaximal dose of ADP can be blocked by prostaglandins I<sub>2</sub>, D<sub>2</sub>, E<sub>1</sub> and E<sub>2</sub>, but not by F<sub>2</sub> $\alpha$ . dbcAMP, a longacting analog of cAMP and forskolin, which blocks the metabolism of cAMP, also block the response to ADP, indicating that the effect is mediated through the increase in cAMP induced by prostanoids. Stimulation of the platelets in the absence of calcium in the surrounding medium markedly blunts the response, but the calcium channel blocker verapamil does not alter the response. This indicates that the increase in free intracellular calcium to a large extent depends on the entry of extracellular calcium through the cell membrane via other mechanisms than through the slow calcium channels. Measurements of the aggregation in response to the above stimuli remain to be done.

The calcium ion is known to be an intracellular second messenger and this study will link the increase in free intracellular calcium with the increase in platelet aggregation known to occur with ADP, as well as with many other stimuli. Drugs that alter the balance of prostanoids and thromboxanes are used or under evaluation in the prevention of myocardial infarction and stroke. Therefore, we feel it is important to demonstrate the effect of perturbations of prostanoids and thromboxanes on platelet function in vitro, thus linking the measurement of a functional parameter with the levels of the intracellular messenger calcium

Preliminary studies on vascular smooth muscle cells have shown basal calcium levels in the range of 110-130 nM. Angiotensin II receptors are expressed in VSMC cultures that have been passed up to 25 times over a period of six months, while the response to bradykinin is markedly reduced after several passages. Bradykinin, which is a vasodilator in whole animal preparations, increases intracellular calcium levels to the same extent as



angiotensin II in our test system. We are now studying the response to other hormones.

Our test system allows us to study the interactions of known vasoactive hormones on intracellular second messengers in a way that comes closer to normal physiologic events than previous methods that use either single cell preparations or cells in suspension. The technique should allow us to determine the effects on intracellular signaling systems during serial passages of VSMC in tissue culture. This should provide us with knowledge of mechanisms underlying essential hypertension on the cellular level.

Publications:

None.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03561-01 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Circulating atrial natriuretic peptides in rats

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Peter Ohman Visiting Associate HE NHLBI

Other: Harry R. Keiser Chief HE NHLBI  
Nadav Zamir Visiting Fellow HE NHLBI

## COOPERATING UNITS (if any)

None.

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.3

## PROFESSIONAL:

.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mechanisms for the release of atrial natriuretic peptide are not clear. We have previously shown that hypophysectomy inhibits the release of ANP following an acute blood volume expansion. The effects of TRH and the importance of thyroid hormones for the basal levels and release of ANP have been evaluated.

The plasma levels of ANP in the basal state and following an acute blood volume expansion are reduced during hypothyroidism but the responsiveness, i.e. the magnitude of increase following the blood volume expansion, is unchanged. Hyperthyroidism does not alter basal or stimulated plasma levels of ANP. Acute TRH administration increases ANP levels in parallel with the increase in blood pressure, but does not alter the responsiveness to acute blood volume expansion. The metabolic state induced by thyroid hormone may modulate the basal levels of ANP but does not alter the release. TRH does not appear to modulate the release of ANP apart from its effects on the sympathetic nervous system.



Project Description:Objectives:

To evaluate the effects of 1) Hypo- and hyperthyroidism on the levels of ANP in the basal state and following an acute blood volume expansion, and of 2) Acute TRH administration on the same parameters.

Methods:

The study has been done in rats with either total thyroidectomy followed by reimplantation of the parathyroids or following thyroxine administration i.p. ANP was measured by radioimmunoassay.

Results and Their Significance:

Hypothyroidism reduced the basal levels of ANP but not the responsiveness to an acute blood volume expansion. Hyperthyroid rats had normal ANP levels both in the basal state and following acute blood volume expansion.

TRH acutely increased ANP levels at the same time as mean arterial pressure increased. Acute blood volume expansion thereafter caused a normal increase on ANP.

This study further clarifies the role of TRH and thyroid hormone in the regulation of basal levels and the release of ANP. It appears that the metabolic state may modulate the basal ANP levels but does not alter the acute release mechanism. TRH has other effects than the release of TSH, but they seem not to include modulation of ANP release.

Publication:

None.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03562-01 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Plasma levels of ANP following inhibition of ACE and kallikrein in the rat.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. Ohman Visiting Associate HE NHLBI

Others: H.R. Keiser Chief HE NHLBI

N. Zamir Visiting Fellow HE NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

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## TOTAL MAN-YEARS:

.3

## PROFESSIONAL:

.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We found that neither the endogenous release of ANP in response to acute volume expansion nor its elimination following a volume load or an intravenous bolus injection of synthetic ANP is markedly altered by inhibition of ACE or kallikrein. We therefore conclude that neither ACE nor kallikrein are key enzymes in the activation or elimination of ANP.

Plasma levels of ANP in the basal state and following acute blood volume expansion are reduced during hypothyroidism but the responsiveness, i.e. the magnitude of increase following acute volume expansion, is unchanged. Hyperthyroidism does not alter basal or stimulated plasma levels of ANP. Acute TRH administration increases ANP levels in parallel with the increase in blood pressure, but does not alter responsiveness to acute volume expansion. Thus the metabolic state induced by thyroid hormone may modulate the basal levels of ANP, but does not alter its release. TRH does not appear to modulate the release of ANP apart from its effects on the sympathetic nervous system.

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Objectives:

To evaluate the effects of inhibition of ACE and kallikrein on 1.) the basal levels of ANP, 2) the levels of ANP achieved by an acute volume load, and 3) the levels of ANP following an i.v. injection of synthetic ANP.

To evaluate the effects of 1) Hypo- and hyperthyroidism on the levels of ANP in the basal state and following an acute blood volume expansion, and of 2) Acute TRH administration on the same parameters.

Methods:

The first part of the study was performed in awake semirestrained rats. The second part was done in rats with either total thyroidectomy followed by reimplantation of the parathyroids or following thyroxine administration i.p. ANP was measured by radioimmunoassay developed in this laboratory and reported previously.

Results and Significance:

The combined inhibition of ACE with captopril and of kallikrein with aprotinin reduced basal levels of ANP. The increase in ANP following an acute expansion of blood volume or i.v. injection of ANP was not altered by the enzyme inhibition. Only a minor prolongation of the elimination of exogenous ANP was noted following inhibition of ACE. No difference in BP response, urinary volume or sodium and potassium excretion was noted.

Inhibition of kallikrein has been a way of investigating the importance of the kallikrein-kinin system in renal excretory functions. It is therefore important to show that inhibition of kallikrein with aprotinin does not produce a major alteration in the dynamics of ANP, which may also affect renal function.

Hypothyroidism reduced the basal levels of ANP but not the responsiveness of ANP to an acute expansion of blood volume. Hyperthyroid rats had normal ANP levels both in the basal state and following acute blood volume expansion.

TRH acutely increased ANP levels at the same time as mean arterial pressure increased. Acute blood volume expansion thereafter caused a normal increase in ANP.

This study further clarifies the role of TRH and thyroid hormone in the regulation of basal levels and the release of ANP. It appears that the metabolic state may modulate the basal ANP levels but does not alter the acute release mechanism. TRH has other effects than the release of TSH, but they seem not to include modulation of ANP release.

Publications:

None.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03563-01 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Endogenous  $\text{Ca}^{2+}$  channel modulator

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
Other:	Enrico Sanna	Visiting Fellow	HE NHLBI
	Gilbert Wright, Jr.	Research Chemist	HE NHLBI
	John W. Daly	Chief	LBC NIDDKD

## COOPERATING UNITS (if any)

Martin Morad	Professor,	Univ. of Pa.
	Dept. of Physiol.	

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.3

## PROFESSIONAL:

.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

3H-Nitrendipine, a  $\text{Ca}^{2+}$ -channel antagonist, was used as a biochemical tool to isolate an endogenous ligand that modulates  $\text{Ca}^{2+}$  channel activity. Brain extracts contain a low molecular weight material (2000 d) that displaces 3H-nitrendipine in a noncompetitive fashion, decreases voltage-dependent and glutamate receptor-dependent  $^{45}\text{Ca}^{2+}$  uptake in cerebellar granule cells and decreases voltage-dependent  $\text{Ca}^{2+}$  channel activity in patch clamped cardiac cells.

543



Project Description:Objective:

Patch clamp studies carried out in various laboratories demonstrated the existence of voltage-dependent and receptor-dependent  $\text{Ca}^{2+}$  channels in cardiac and smooth muscle cells and in neurons. Studies on the structure and function of  $\text{Ca}^{2+}$  channels are in progress due to the availability of drugs and toxins that specifically interact with molecular sites of the  $\text{Ca}^{2+}$  channel complex. Reports in the literature demonstrated that binding sites of dihydropyridine, a potent  $\text{Ca}^{2+}$  channel antagonist, were up-regulated in sympathetically denervated heart, and in brain tissue of morphine-tolerant mice or after alcohol withdrawal. This finding suggests that the binding sites of dihydropyridines may be recognition sites for endogenous  $\text{Ca}^{2+}$  channel modulators.

Methods:

Measurement of displacing activity of  $^3\text{H}$ -nitrendipine binding was used to monitor the presence of an endogenous ligand in aqueous extracts of heart or brain tissue. The bulk proteins in the supernatant fraction were precipitated with TCA (3% final concentration) followed by four washes with 4 V of ether and lyophilization of the aqueous supernatant fraction. The dried material was redissolved and filtered on Biogel-P10 column (0.9 x 60 cm, equilibrated in 0.1 N acetic acid). The fractions containing  $^3\text{H}$ -nitrendipine-displacing material were desalted on a Silica-SEPPAK cartridge and eluted by a stepwise gradient of methylene chloride + methanol. The fractions containing nitrendipine-displacing material were further purified by HPLC technique using a Partisil-SCX-10 or a partisil-5 column.

Chemical Characterization of the active material involves:

- 1) Thin layer chromatography; and,
- 2) Mass spectrometry.

Characterization of Physiological Functions:

- 1) Measurement of  $^{45}\text{Ca}^{2+}$ -uptake in primary cultures of cerebellar granule cells; and,
- 2) Modulation of  $\text{Ca}^{2+}$  channel activity in isolated rat heart cells and cultured neuronal cells by patch clamp technique.

Results:

Nitrendipine-displacing material coeluted with a molecular weight marker of 1200 d off of a Biogel-P10 column. The active material is heat-stable, soluble in  $\text{H}_2\text{O}$ , methanol or ethanol and insoluble in chloroform or ether. When fractions eluted off the partisil SCX-10 column were chromatographed on TLC plates, two UV-sensitive spots were discernable. The spot containing nitrendipine-displacing activity was also stained by iodine and ninhydrin spray reagents. Acid hydrolysis (in constantly boiling





HCl at 155°C for 30 min. or at 120°C for 48 hrs) abolished the nitrendipine-displacing activity and the inhibitory effect on veratridine-stimulated  $^{45}\text{Ca}^{2+}$  uptake in cultured cerebellar granule cells. Patch clamp studies carried out on rat cardiac myocytes showed that the nitrendipine-displacing material inhibited the voltage-dependent  $\text{Ca}^{2+}$  channel activity. Preliminary evidence indicates that this effect is mediated by accelerating the gating mechanism.

Future research is aimed at the chemical characterization of this endogenous modulator for  $\text{Ca}^{2+}$  channels, and to establish a procedure for measurements that will allow studies on the physiological function and regulation of this material.

Publications:

- 1) Hanbauer, I. and Sanna, E.: Endogenous modulator for nitrendipine binding sites. Clinical Neuropharmacology 9: 220-222, 1986.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03564-01 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulatory mechanisms for adrenal chromaffin cell secretion.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Ingeborg Hanbauer Pharmacologist HE NHLBI

## COOPERATING UNITS (if any)

Fidia-Georgetown Institute for Neuroscience, Washington, DC (A. Guidotti)

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLRI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.3

## PROFESSIONAL:

.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The chromaffin cells of canine adrenal medulla served as a model to study the regulation of the cholinergic nicotinic receptor. The role of GABAA receptors in the regulation of the release of catecholamine and met-enkephaline-like (ME) peptides was studied in situ by autoperfusion of the adrenal gland. Infusion into the adrenal gland of GABAA receptor antagonists increased the release of catecholamines and ME-peptides into the adrenal effluent blood. This release was attenuated by GABAA receptor antagonists, but not by hexamethonium, naloxone or prior splanchnic nerve transection. In contrast, the extent of release of catecholamines and ME-peptides elicited by splanchnic nerve stimulation was decreased by GABAA receptor agonists and facilitated by GABAA receptor antagonists. These data suggest that the release of these neurotransmitters may be triggered by direct stimulation of GABAA receptors, presumably causing membrane depolarization by a burst of chloride channel opening. This type of depolarization may be responsible for obtunding the subsequent depolarizing effect of nicotinic receptor stimulation.

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Projective Description:Objectives:

Signal transduction at the nicotinic receptor in chromaffin cells appears to be modulated by cotransmitters. Cotransmitters, like primary transmitters, are synthesized and stored in nerve cells and can be released by depolarization of these cells. Functionally cotransmitters appear to either amplify or reduce signal transduction elicited by physiological stimuli. It has been suggested that in chromaffin cells multiple chemical signals including GABA, enkephalins, substance P, and neuropeptide Y, may modulate nicotinic receptor function by alteration of the transmitter recognition sites, the coupling of the recognition sites to the transducer or by acting on ion channels. Similarly to brain neurons, the GABA receptor on adrenal chromaffin cells appears to be functionally associated with chloride channels. The goal of the present project is to study the interrelationship between acetylcholine and GABA receptors. Specific attention is paid to the modification elicited by GABA receptor agonists or antagonists of the catecholamine release elicited by direct stimulation of nicotinic receptors or by electrical stimulation of the splanchnic nerve.

Methods:

Studies of the release of catecholamines and ME-peptides from canine adrenal gland include measurements of the amount of epinephrine and norepinephrine released into the adrenal effluent blood that was collected through an indwelling catheter in the inferior vena cava situated at the level of the lumbar adrenal vein. Transection of the splanchnic nerves was carried out below the diaphragm on the day of the experiment or two weeks before. The distal stump of the splanchnic nerve was electrically stimulated at 10 Hz and various voltages. Saline or drugs to be tested were infused into an arterial pouch that irrigated solely the adrenal gland. The catecholamines were extracted from plasma and the content was measured by HPLC technique using a reverse phase column (Biosil ODS10) and an electrochemical detector.

The ME-peptides were extracted with 2 N acetic acid and the supernatant fraction was filtered through a C<sub>18</sub> SEPPAK cartridge previously equilibrated with H<sub>2</sub>O. The ME-peptides were eluted with 3ml 6% 2-propanol. ME immunoreactivity was determined by radioimmunoassay. The recovery of added met<sup>5</sup>-enkephalin standard ranged from 70% to 85%.

Major Findings:

GABA, muscimol and THIP (GABA<sub>A</sub> receptor agonists) increased the release of catecholamines from adrenal chromaffin cells, while Baclofen (GABA<sub>B</sub> receptor agonist) was ineffective. The increased catecholamine release elicited by GABA<sub>A</sub> receptor agonists was blocked by bicucullin (GABA<sub>A</sub> receptor antagonist) but was not prevented by hexamethonium, naloxone or prior splanchnic nerve transection. These data suggested that the increased catecholamine release into the effluent adrenal blood was not caused by a transsynaptic mechanism but rather resulted from direct stimulation of GABA<sub>A</sub> receptors located on chromaffin cells.



Occupancy of GABA receptors is associated with an opening burst of chloride channels that may lead to the depolarization of chromaffin cell membranes. Hence, the release of endogenous GABA that is present in chromaffin cells may depolarize a population of chromaffin cells determined by the chloride current. Additional data showing that the catecholamine release elicited by electrical stimulation of the splanchnic nerve was attenuated by GABA<sub>A</sub> receptor agonists and was facilitated by GABA<sub>A</sub> receptor antagonists indicated that GABA may alter the responsiveness of nicotinic receptors on chromaffin cells to ongoing cholinergic transmission. In fact, the present data suggest that GABA<sub>A</sub> receptor-mediated depolarization of chromaffin cells may be responsible for obtunding the subsequent depolarizing effect of nicotinic receptor stimulation.

#### Proposed Course:

Met<sup>5</sup>-enkephalin-like peptides and neuropeptide Y are similar to catecholamines in that they are present in adrenal chromaffin cells and released by nicotinic receptor stimulation. Experiments are in progress to determine whether GABA modulates the release of these neuropeptides.

#### Significance to Biomedical Research:

This research project focuses on the regulation of the release of catecholamines and neuropeptides from adrenal chromaffin cells into the blood circulation. This project introduces a novel mechanism for modulating the release of these transmitters. Since catecholamines, enkephalins and other neuropeptides secreted from adrenal gland participate in a number of physiological mechanisms including the regulation of cardiovascular function, the development of new drugs such as analogs to THIP or muscimol may be useful to reduce the adrenal participation during stress responses.

#### Publications:

Kataoka, Y., Fujimoto, M., Alho, H., Guidotti, A., Geffard, M., Kelly, G. D. and Hanbauer, I.: Intrinsic GABA receptors modulate the release of catecholamines from canine adrenal gland. J. Pharmacol. Exper. Ther. 239: 584-590, 1986.

Fujimoto, M., Kataoka, Y., Guidotti, A. and Hanbauer, I.: Effect of GABA<sub>A</sub> receptor agonists and antagonists on the release of enkephalin-like peptides from dog adrenal gland. J. Pharmacol Exper. Ther. (in press), 1987.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03565-01 HE

## PERIOD COVERED

Oct. 1, 1986 through Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biosynthesis, distribution and biological function of substance P and its receptors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mei-Lie Swenberg

Research Chemist

HE NHLBI

Other: Rita Liu

Asst. Professor

USUHS

## COOPERATING UNITS (If any)

None.

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Localization of substance P(SP) receptor was conducted on the midbrain of adult Sprague-Dawley (SD) and spontaneously hypertensive rats (SHR). Significant difference of 79.9 and 32.2% lesser in density of the receptor distribution was observed in zona inserta and lateral hypothalamus respectively in SHR than in Sprague Dawley.

The chemical and physical analysis of the receptor proteins isolated and purified from olfactory bulb and intestine reveals differences in the isoelectric points and in binding displacement with SP antibodies. Further investigation is underway to examine both chemical and functional differences in the different SP receptor types.

SP and SP receptors were found densely and uniformly distributed in uterus of Sprague-Dawley rats. The receptors appeared to migrate during pregnancy toward the basal endometrium. The specific binding/mg wet tissue decreased as the pregnancy progressed (2,200 cpm/mg in nonpregnant uterus to 1,000/mg of uterus at full term). The disproportional growth in the uterus and its SP receptors would be of benefit in decreasing pain perception during pregnancy.

SP was found densely distributed in atrial muscle but few SP receptors were observed under the conditions examined.

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Project Description:Objectives:

Substance P (SP) serves as a neurotransmitter in both the central and peripheral nervous systems. There is evidence that SP also plays a role in blood pressure regulation. It is important to know how this neuropeptide interacts with its receptors and how they function in the system. In order to achieve these goals, there are several approaches that could be pursued. These include:

- 1) Isolation, purification and characterization of the receptor molecule;
- 2) Derivation and preparation of new probes for studying the interaction of the peptide with its receptors, such as anti-idiotypic antibodies, antireceptor antibodies (polyclonal or monoclonal);
- 3) Determination of developmental pattern for SP and its receptors and the correlation of these with changing functions;
- 4) Studies on the primary sensory system might derive a mechanism for understanding the role of SP which is a major neurotransmitter in the system.

Methods:

Localization of receptors was performed on freshly sectioned frozen tissue, with Bolton Hunter iodinated SP (BHSP) in the presence of protease inhibitors. Membrane receptor binding was carried out with  $^3\text{H}$ -SP and the freshly prepared membranes.

SP receptors were extracted with Triton X-100 or 3[(3-cholamidopropyl) dimethyl ammonio]2-hydroxy-1-propanesulfonate (Chapso) and purified with wheat-germ lectin affinity column chromatography and elution with N-acetylglucosamine.

Isoelectric focusing of purified receptor proteins was performed with phastgel acrylamide or agarose electrophoresis and sucrose gradient isoelectric focusing.

Molecular weight determination of the protein was carried out by HPLC on TSK 3,000 and 4,000 analytical columns in Tris buffer (pH 7.4).

Antibodies were raised in New Zealand white rabbits, immunized with purified receptors.

Results and Their Significance:

- 1) The concentration of SP receptors in the various parts of the mid brain of SHR was the same as that of SD except in zona inserta and lateral hypothalamus which were reduced by 80% and 32% respectively in SHR. This phenomena is currently under study in Wistar Kyoto rats.



- 2) Dense and uniform distribution of SP was observed in atrial muscle fibers but only very faint SP receptor distribution was found under the experimental conditions examined.
- 3) The chemical composition of receptor protein from olfactory bulb is different from that of intestine; IPs are different.

SP receptors were found densely distributed in the endometrium of pregnant and nonpregnant rat uteri. As pregnancy progressed, the specific binding of BHSP/g tissue decreased and the receptors migrated toward the basal endometrium to form a ring. SP was found distributed in the entire uterus, both pregnant and nonpregnant, but most densely distributed in all the endothelium of blood vessels, the epithelium of endometrium mucosa, muscle, glandular cells, nerve fibers and outer uterine wall. These results suggest that SP and its receptors may play an important role in uterine physiology, and possible pain regulation during the pregnancy.

The peptide substance P appears to be an important neurotransmitter in the CNS and peripheral nervous systems. Previous studies in our laboratory have demonstrated the nature of the interaction of this neuronal system with the dopamine and serotonin neuronal systems. The current experiments were designed to further understand both the development of the system and its interaction with the endocrine system. The work is directed at understanding the role of SP in cardiovascular control and other physiological systems.

#### Proposed Course:

- 1) Further comparison of SP receptors in midbrain of WKY & SHR might indicate the importance of SP system in blood pressure regulation of these two genetically different animals.
- 2) Further investigation of SP and its receptors in the cardiovascular system might be beneficial in understanding its role in blood pressure regulation.
- 3) With purified receptor available, antibodies could be prepared for use in the study of the regulation and function of SP in cardiovascular and sensory nervous systems as well as in the neuroendocrine system.
- 4) Hybridoma cell lines will be obtained to produce monoclonal antibodies against isolated receptors from CNS or peripheral nervous system.
- 5) Continue the studies of possible new functions of SP in prenatal and postnatal development.
- 6) Studies will be conducted to examine the possibility on development of immunological treatment for neuropeptidergic deficiency or disorder.

#### Publication:

- 1) Swenberg, M.L., Buck, S. and Lovenberg, W. Development of an anti-idiotypic antibody that blocks Substance P primary antibodies and Substance P membrane binding. Brain Research. In press, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03566-01 HE

## PERIOD COVERED

Oct. 1, 1986 to Sept. 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Apparent MgH-ATPase activity and proton transport in adrenergic vesicles in situ.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. Bogdanski

Pharmacologist

HE NHLBI

Others: J. Jacobs

Bio. Lab. Tech.

HE NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Hypertension-Endocrine Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

1.75

## OTHER:

.25

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A Ca<sup>++</sup>-dependent secretion of norepinephrine ([<sup>3</sup>H]NE) was evoked in adrenergic nerves in rat heart ventricle slices incubated in a modified Krebs-HCO<sub>3</sub> medium containing choline Cl as the replacement for NaCl (Ch<sup>+</sup> -Ca<sup>++</sup>). Exogenous ATP inhibited secretion, suggesting that the nucleotide stimulated a rapid uptake of [<sup>3</sup>H]NE in vesicles proximate to the axolemma. Lithium<sup>+</sup> ion, a known inhibitor of NE uptake dependent upon Mg<sup>++</sup>-ATPase activity in vesicles (but not ouabain), prevented the response to ATP in axoplasmic vesicles (Bogdanski 1983, 1986). This report indicates that other known inhibitors of uptake in isolated vesicles also inhibited the response to ATP in axoplasmic vesicles. Included were the inhibitors of Mg<sup>++</sup>-ATPase activity N-ethylmaleimide (NEM) and dicyclohexylcarbodiimide (DCCD) and the proton transporters 2,4-dinitrophenol (2,4-DNP 1.0 mM) and chlorpromazine (CPZ). Potassium ionophores (valinomycin and 2,4-DNP 0.1 mM, and nigericin) a carrier blocker (reserpine) and a pH neutralizing reagent for vesicles (NH<sub>3</sub> from ammonium sulfate in solution) were also effective. The inhibitors also increased the rate of depletion of stored NE and its deamination in nonsecreting nerve endings incubated in Krebs-HCO<sub>3</sub> (KRB) medium. Valinomycin stimulated uptake in the presence of ATP. It is suggested that mechanisms of uptake of NE in isolated vesicles apply to the axoplasmic vesicles as well. Thus, the activity of Mg<sup>++</sup>-ATPase drives proton transport to establish the electrochemical gradients of H<sup>+</sup> which drive the transport of NE. Isolated adrenal medullary vesicles in the presence of ATP and Cl<sup>-</sup> translocate a sufficient number of ions to establish a pH gradient and eventually cause osmotic lysis of the vesicle. By contrast exocytosis in axoplasmic vesicles was not apparent biochemically.

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Project Description:Objective:

To study mechanisms of catecholamine retention in vesicles in situ.

Methods:

Slices of heart ventricle labelled with tritiated NE were preincubated for 90 min in beakers containing 20 ml of Krebs-HCO<sub>3</sub> medium. They were then transferred to various media for incubation.

Preincubated slices were transferred to beakers containing 20 ml of either Krebs-bicarbonate medium supplemented with 0.3% bovine serum albumin (KRB-BSA) or  $\text{Ch}^+-\text{Ca}^{++}$  likewise supplemented ( $\text{Ch}^+-\text{Ca}^{++}$ -BSA). The transfer began the incubation phase of the experiment. After 60 and 80 min, 0.5 ml aliquots of the media were transferred to vials for liquid scintillation counting of [<sup>3</sup>H] activity.

After the aliquots were transferred at 80 min, groups of slices were transferred to beakers containing 20 ml of fresh  $\text{Ch}^{++}-\text{Ca}^{++}$  BSA. Some of these media contained either disodium ATP to inhibit neurosecretion or disodium ATP and a known inhibitor of the uptake dependent upon  $\text{Mg}^{++}$ -ATPase activity in isolated vesicles. Aliquots of media (0.5 ml) were transferred to scintillation vials for counting [<sup>3</sup>H] at the 100 min time point. The slices were blotted, weighed and homogenized in 5.0 ml of 0.4 N perchloric acid. After centrifugation, 0.5 ml of the clear tissue extract were transferred to scintillation vials.

Results:

Effects of  $\text{Mg}^{++}$ -ATPase inhibitors on  $\text{Ch}^+-\text{Ca}^{++}$ -stimulated secretion in adrenergic nerve endings. The enzyme inhibitors N,N'-dicyclohexylcarbodiimide (DCCD) and N-ethyl maleimide (NEM) completely prevented the inhibitory response to ATP. The enzyme inhibitors also depleted [<sup>3</sup>H]NE in tissue that were incubated in KRB. However, the prevention of the response to ATP began more rapidly and was complete. This rapid effect of the inhibitors suggested that the inhibitors specifically affected the uptake process rather than acting secondarily through a generalized metabolic inhibition.

Effects of cation transporters and ionophores on  $\text{Ch}^{++}-\text{Ca}^{++}$ -stimulated neurosecretion of [<sup>3</sup>H]NE in rat heart ventricle slices.

1. The effects of 2,4-dinitrophenol (2,4-DNP). This proton transporter dissipates electrochemical energy stored in the form of proton gradients across membranes. In a concentration of 1.0 mM it prevented a part of the inhibitory response to ATP but exerted little effect on the secretion evoked by  $\text{Ch}^+-\text{Ca}^{++}$  alone or by KRB. These findings suggested that 2,4-DNP actually prevented the response to ATP rather than depleting NE elsewhere in the nerve ending.

2. The effects of 2,4-DNP and valinomycin on  $\text{Ch}^+-\text{Ca}^{++}$ -stimulated neurosecretion in rat heart ventricle tissue slices. Valinomycin is a  $\text{K}^+$  ionophore which may not by itself inhibit the uptake of NE by vesicles. In the presence of a low concentration of 2,4-DNP the combination of uptake inhibitors may act by inducing an exchange of extracellular  $\text{K}^+$  for intracellular  $\text{H}^+$ , thus reducing the electrochemical gradient of  $\text{H}^+$ .



In heart slices, valinomycin (3  $\mu\text{g/ml}$ ) in the medium together with ATP (2 mM) actually increased retention. When 2,4-DNP (0.1 mM) was also present, the inhibitors in combination partly blocked the active retention.

3. The effects of nigericin on the  $\text{Ch}^+-\text{Ca}^{++}$ -stimulated secretion of  $[^3\text{H}]\text{-NE}$  in rat heart ventricle slices. This ionophore reduces the electrochemical gradients of  $\text{H}^+$  across the vesicle membrane by inducing an electrically neutral exchange of  $\text{H}^+$  and  $\text{K}^+$  across biological membranes. As expected, nigericin prevented the inhibitory response to ATP in  $\text{Ch}^+-\text{Ca}^{++}$ -stimulated adrenergic nerve endings. The ionophore showed no effect on the  $\text{Ch}^+-\text{Ca}^{++}$ -stimulated secretion in the absence of ATP but inhibited the retention of NE in tissue incubated in KRB (Table 3).

4. The effects of chlorpromazine (CPZ) on the  $\text{Ch}^+-\text{Ca}^{++}$ -stimulated secretion of  $[^3\text{H}]\text{NE}$  in rat heart ventricle slices. Chlorpromazine lowers the electrochemical gradient of  $\text{H}^+$  across the vesicle membrane. In concentrations of 1 to 40  $\mu\text{M}$ , CPZ prevented ATP from exerting its maximal inhibitory effect on secretion. In concentrations of up to 40  $\mu\text{M}$  in KRB, CPZ showed no inhibitory effect on retention within the 20 min time period used for these studies.

5. The effects of ammonium sulfate on the retention of  $[^3\text{H}]\text{NE}$  in rat heart ventricle slices. The ammonia ( $\text{NH}_3$ ) derived from the spontaneous decomposition of the salt in solution neutralizes the pH gradient developed during proton transport driven by  $\text{Mg}^{++}\text{-ATPase}$  activity in the presence of a permeant anion like  $\text{Cl}^-$ . In rat heart ventricle slices,  $\text{NH}_3$  completely prevented the inhibitory response to ATP. When present in concentrations of 1.0 to 30 mM, the salt induced a concentration dependent depletion of  $[^3\text{H}]\text{NE}$  in tissues incubated in KRB. The release began rapidly and was massive even when the concentration of salt was only 7.5 mM.

The proportions of  $[^3\text{H}]\text{amines}$  and  $[^3\text{H}]\text{deaminated metabolites}$  in the tissues and media. After the various uptake inhibitors had blocked the inhibitory response to ATP, the quantity of  $[^3\text{H}]\text{amines}$  in the media was greater than in those media containing no inhibitors. These amines must have been mobilized, secreted, and recovered close enough to the axolemma to avoid deamination by tissue MAO.

Tissues that were incubated in KRB, however, were not stimulated to secrete. Amines mobilized by the various uptake inhibitors in vesicles located deeper in the axoplasm were deaminated by intraneuronal MAO. Lithium<sup>+</sup>, DCCD, nigericin and  $\text{NH}_3$  all increased the quantity and the proportion of  $[^3\text{H}]\text{deaminated metabolites}$  of  $[^3\text{H}]\text{NE}$  recovered in the medium. By contrast, the inhibitors slightly increased the quantity of  $[^3\text{H}]\text{amines}$  released, indicating that only a small fraction of the mobilized amines had direct access to the axolemma in the non-stimulated nerve ending.

#### Significance to Biomedical Research and Institute Programs:

In the model secretory system described above, the apparent inhibitory effect of ATP on the secretion of NE has been interpreted to be mediated by the active uptake of the transmitter (Bogdanski, 1983, 1986). This active retention of transmitter is prevented by biochemical reagents that display diverse biochemical activity and molecular structure. These reagents are known to block the uptake of NE dependent upon  $\text{Mg}^{++}\text{-ATPase}$  activity in vesicles isolated from the adrenal medulla (Johnson and Scarpa, 1984) and brain (Toll and Howard, 1978; Slotkin et al., 1978). Thus, biochemical



mechanisms operating in isolated vesicles may also operate in vesicles in the modified axoplasm of  $\text{Ch}^+$ - $\text{Ca}^{++}$ -stimulated nerve endings. Using the metabolism of tissue NE as a criterion for the inability of vesicles to retain NE, the response to the same reagents indicates that these mechanisms also operate in the more normal axoplasm of nerve endings incubated in KRB-BSA.

The findings described above represent further support for the biochemically defined vesicular axolemmal neurosecretory and transmitter recovery unit. This unit may account for the prolonged uptake of NE (active retention) when the concentration of  $\text{Cl}^-$  is in the physiological range (Bogdanski, 1983). By contrast, isolated vesicles ultimately respond to ATP and  $\text{Cl}^-$  by a release of amines, osmotic swelling and lysis caused by the uptake of large quantities of  $\text{H}^+$  and  $\text{Cl}^-$ . This response is the basis of a theory of exocytosis (Pazoles, Creutz, Ramu and Pollard, 1980). This Laboratory does not support the theory of exocytosis.

The vesicular uptake mechanisms described above may represent a normal mechanism for the retention of catecholamines by vesicles in vivo as discussed in a previous report (Greenspan, Aronoff and Bogdanski, 1970 and Bogdanski, 1986).

#### Future Course of Project:

This report concerns work which is part of an ongoing project on mechanisms of storage and release of NE in peripheral adrenergic nerve endings. The incubation media used enable us to study the metabolic requirements for the retention of NE in vesicles in the axoplasm. Whereas the basic mechanisms, which are similar to those previously discovered in mitochondria, have been observed in isolated vesicles, the requirement for the electrical component of the electrochemical gradient of  $\text{H}^+$  can only be observed in media lacking a permeant anion. However, the physiological environment of vesicles in the axoplasm is never lacking in  $\text{Cl}^-$ , a permeant anion. When  $\text{Cl}^-$  is present in isolated vesicles, the ultimate response to proton pumping is lysis of the vesicles. By contrast axoplasmic vesicles actively retain NE during prolonged exposure to ATP and  $\text{Cl}^-$ . Further studies with ammonium sulfate, proton transporters, reserpine and other biochemical reagents can solidify and expand our knowledge of processes of retention by vesicles in situ. For example, preliminary studies have indicated an electrolyte requirement for retention. No such requirement has been described for isolated vesicles. Other studies contemplated include the retention of NE by genetically hypertensive rats. The HPLC method of analysis has been used in some of these studies, obviating the need for radioactive compounds.

#### Publications:

Vesicular site of action of lithium ion in choline-calcium stimulated adrenergic nerve endings of rat heart. Neuropharmacology. 25: 1327-1334, 1986.



ANNUAL REPORT OF THE  
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE  
October 1, 1986 to September 30, 1987

Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that support and control transport and metabolism.

Isolated segments of renal tubules.

In order to understand kidneys at a cellular and molecular level the functions of the different types of epithelial cells must be identified. Progress in this direction has relied heavily on the direct study of individual nephron segments. Each nephron segment has a different cell morphology and function. An important method (which originated in this laboratory) for directly studying the nephron segments is to dissect them and perfuse them individually in vitro. The findings of Knepper, Burg, and their colleagues during the past year using this method are as follows:

Acid excretion, which is one of the vital functions of the kidney, depends mainly on the rates of urinary ammonium and bicarbonate excretion. The excretion of these ions depends in turn on the integration of metabolism and transport in numerous nephron segments.

Previous studies had demonstrated absorption of ammonium ions by isolated perfused thick ascending limbs. The new studies were carried out to determine whether this absorption occurs passively (driven by the lumen positive voltage), or whether ammonium is actively reabsorbed. Isolated perfused medullary thick ascending limbs from rabbits were used. When the transepithelial voltage was reversed experimentally, ammonium absorption decreased somewhat, but a large component persisted, indicating that ammonium ions are absorbed by the thick ascending limb both by active and passive (voltage driven) transport.

When there is luminal carbonic anhydrase activity, as previously found in proximal convoluted tubules, acidification results in minimal lowering of the pH (no pH disequilibrium) and a maximal rate of acidification. In order to test this relation in cortical collecting ducts, they were isolated from rabbits and perfused with a pH-sensitive dye 1,4-DHPN that measured luminal pH. There was a significant acidic disequilibrium pH, which dissipated when carbonic anhydrase was added to the lumen. The effective rate constant for carbonic anhydrase





dehydration in the lumen was measured by a newly devised technique. It was equal to the uncatalyzed rate, indicating complete absence of carbonic anhydrase activity. Because of the pH disequilibrium low rates of acidification in cortical collecting ducts decrease the lumen pH to low levels which enhances ammonia secretion into the urine.

Effects of hormones on collecting ducts. The collecting ducts, which are the last segments of the nephron, are responsible for much of the control of urinary excretion. They accomplish this by changing transepithelial transport in response to hormones.

Vasopressin (antidiuretic hormone) affects both water and urea excretion. The parts of the inner medullary collecting ducts (IMCDs) first traversed by the tubule fluid (initial IMCDs) and last traversed by the tubule fluid (terminal IMCDs) were studied separately. Vasopressin increased the permeability both to urea and water in the terminal IMCD, but increased only water permeability in the initial IMCD. Based on mathematical modelling, this pattern of regulation of permeability is most efficient for the urinary concentrating mechanism.

Changes in intracellular calcium and cyclic AMP have both been implicated as second messengers for vasopressin action in various cells. The relative importance of these two systems was studied in rat terminal IMCDs. Vasopressin increased both intracellular calcium activity (using Ca-sensitive dye INDO-1) and cyclic AMP accumulation (using radioimmunoassay). Both effects occurred within 1 minute, as did the increase in urea permeability. The cAMP accumulation and urea permeability increase both occurred at the same low vasopressin concentration, but rise in intracellular calcium occurred only at much higher concentrations of vasopressin. 8-bromo-cAMP increased urea permeability without affecting intracellular calcium. The conclusions are that cyclic AMP mediates the vasopressin-induced increase in urea permeability, and that the second messenger role of calcium, if any, remains to be discovered.

Atrial natriuretic factor (ANF) has large effects on urinary water and solute excretion, but there is little direct information about its specific effects on nephron segments. Previously, ANF was found to bind to inner medullary collecting ducts and increased cyclic GMP production by activating a membrane bound guanylate cyclase. The new studies were aimed at testing for physiologic effects of ANF on permeability to water, urea, chloride, or sodium in isolated perfused rat inner medullary collecting ducts. ANF decreased osmotic water permeability in tubules exposed to submaximal concentrations of vasopressin, but had no effect on osmotic water permeability when vasopressin was absent or when vasopressin was present in supramaximal concentrations. ANF also slightly decreased the apparent chloride permeability of the epithelium, but had no effect on urea permeability or sodium permeability. It did not affect cyclic AMP accumulation by isolated IMCDs either in the presence or absence of the phosphodiesterase inhibitor IBMX.



## Transport in model planar epithelia.

The transporters in some planar epithelia such as toad bladders, toad skins, and Necturus gall bladders are similar to those in parts of the nephron. These planar epithelia are easier to manipulate than individual nephrons, making them valuable models for studying the transporters. Spring and his colleagues studied solute and water transport by Necturus gall bladders and toad skins. They developed and used a combination of light microscopic, video, computer, and electrophysiologic methods to study cell volume and intracellular ions. Regulation of intracellular NaCl was studied in Necturus gallbladder. Chloride exit from the cells was found to involve sodium and bicarbonate and was probably due to a basolateral cotransport system. Such a cotransporter has been identified in many epithelial cells and seems to play a significant role in both transepithelial transport and in the regulation of cell pH.

The route of NaCl transport across toad skin was studied, using optical techniques. A minority cell type, the mitochondria rich cell, was responsible for the passive movement of chloride across the skin epithelium. This cell had a voltage dependent channel for chloride. Very similar appearing cells are present in many other tissues, including kidney, urinary bladder, salivary gland, and fish gill. The information obtained from toad skins will be of general use in understanding this kind of epithelial cell.

Improved techniques were developed for fluorescence microscopy of living cells. The improvements involve a newly developed illumination and detection system that provides high sensitivity for the detection of fluorescent dyes in cells. Dyes are currently available that can be used to measure pH and calcium concentration. The system is so sensitive that it visualized single fluorescent VSV viruses, as they infected renal epithelial cells and entered various cellular compartments.

Vasopressin increases the water permeability of specific tubule segments and other urinary tract epithelia like toad bladders. The increase results from fusion with the epithelial urinary plasma membrane of aggrephores, which are intracellular vesicles, believed to contain water channels. Handler and his colleagues isolated aggrephores in order to study the molecular structure of the putative water channels. They used a strategy based on the response of aggrephores to vasopressin to label the proteins in aggrephores with radioactive iodine. Then, they purified the labeled aggrephores by a density shift procedure. Monoclonal antibodies were raised against the proteins in the purified aggrephores. Some antibodies were identified that bound specifically to aggrephores in intact cells and also bound to isolated aggrephore proteins. Thus, considerable progress has been made towards isolating the water channels.



### Inner ear.

Endolymph is the fluid which fills the inner ear membranous labyrinth. It is unique among extracellular fluids in its composition, KCl being virtually the only salt. The high K content of endolymph is an absolute requirement for hearing and equilibrium. Nothing was previously known about the site or mechanism of endolymph production. Spring and colleagues developed a method to dissect the semicircular canals of the ear of the dogfish shark, *Squalus acanthias*, and study them in vitro. The isolated semicircular canals secreted endolymph at a rapid rate. The mechanism of endolymph production was determined from the effects of drugs known to affect specific transport processes. This is the first model system in which endolymph formation can be directly studied, and it may constitute an important breakthrough for the physiology of hearing and clinical otolaryngology

### Cell culture of epithelia.

Although the technique of perfusing kidney tubules in vitro has provided an overall description of their transport properties, it has been difficult to extend the studies to subcellular and molecular levels. Chemical and physical methods for studying transport require much larger amounts of homogeneous tissue than are present in single tubules. Handler, Burg and their colleagues have been using cultures of renal epithelial cells to overcome this difficulty. In addition, epithelia in culture can be readily maintained for prolonged periods of time under conditions not obtainable in intact tissues, and the cultures are more amenable to study by number of the techniques of cell and molecular biology.

Differentiated lines of cells from cortical collecting ducts (CCD). Single tubules were dissected from rabbit kidneys and seeded on sheep amnions in special media. No continuous lines of CCD have resulted as yet, but epithelia are growing for as long as one year and into the fifth passage. The following differentiation persisted. Like native CCD, the tissue culture epithelia had a transepithelial voltage, inhibited by amiloride and stimulated by vasopressin. The CCD epithelia in culture also expressed sodium absorption, potassium secretion, and bicarbonate absorption. In addition, lactate produced by the cells was excreted only to the basolateral medium, while protons from lactic acid were excreted to the apical medium. The latter finding may point to a possible source of protons for urinary acidification.

Osmolytes. Bacterial, plant, and invertebrate animal cells are known to accumulate compatible, osmotically active, organic intracellular solutes when their environment became hyperosmotic. These "osmolytes" help maintain the intracellular milieu because they do not perturb vital intracellular macromolecules, in contrast to sodium and potassium salts which in abnormally high concentrations



do perturb macromolecules. Most mammalian tissues are not normally hyperosmotic and presumably do not express osmolytes. The exception is the renal inner medulla which is hyperosmotic because of the renal concentrating mechanism. In previous studies the principal osmolytes in rat and rabbit inner medullas were identified as sorbitol, inositol, glycerophosphorylcholine, and betaine.

A continuous cell line (GRB-PAP1) was established from rabbit papillary pelvic epithelial cells and studied for osmolytes. These cells were previously found to accumulate sorbitol in hyperosmotic medium. The sorbitol was synthesized from glucose, catalyzed by aldose reductase (AR). Following hyperosmolality, the activity of AR increased because of increased enzyme protein. The new studies concerned the nature of the stimulus that induces AR. AR activity and AR protein did not increase until 12 to 24 hours after the osmolality was increased and were maximum after 3 to 4 days. The response was triggered by adding raffinose (a large molecule) which shrank the cells and increased cell sodium and potassium. In contrast urea and glycerol (small size) did not cause any response and mannitol and sorbitol (intermediate size) caused an intermediate response. Elevation of cell Na by ouabain did not trigger the response. Therefore, elevated cell K or reduced volume are the most likely signals.

The time course for induction of AR (days) was longer than the expected time course of changes in inner medullary osmolality, following, for example, anti-diuretic hormone or diuretics. Therefore, the effect of acute changes in osmolality on sorbitol metabolism was studied in fully induced cells. GRB-PAP1 cells were grown in hyperosmotic medium, then the osmolality was abruptly decreased to isosmotic. AR remained elevated, decreasing by half only after 7 days. Cell sorbitol level fell rapidly, however, due to efflux of sorbitol into the medium. The permeability to sorbitol was very low as long as the cells remained in hyperosmotic medium, but increased at least 200-fold following decrease in osmolality. A large fraction of the sorbitol left the cells within 15 minutes. Thus, sorbitol level in adapted cells is regulated by at least two mechanisms: 1) rapid change in sorbitol efflux and 2) slow changes in AR.

The GRB-PAP1 cells accumulated a high concentration of sorbitol, but not of other osmolytes, in hyperosmotic medium. Therefore, other lines of cells were screened for survival in hyperosmotic medium. MDCK, LLC-PK1, LLC-PK3, A6, and GRB-MAL1 survived in high NaCl and/or urea concentrations. The cells adjusted their volumes and sodium and potassium levels back towards normal and accumulated osmolytes. The osmolytes were inositol, glycerophosphorylcholine, and betaine in the MDCK and LLC-PK1 lines. Only sorbitol (and to a much lesser extent inositol) was accumulated by GRB-MAL1 cells. These cell lines provide models to investigate the mechanism and control of accumulation of the osmolytes other than sorbitol.

Metabolism associated with solute transport.





A large fraction of the metabolism of renal epithelial cells is utilized to produce energy for transepithelial transport. Balaban and his co-workers have been using the noninvasive techniques of nuclear magnetic resonance and optical spectroscopy to investigate the general mechanisms that coordinate cellular energy metabolism with work in the kidney and heart.

Relation between work and energy production in the heart. High energy phosphates were measured by  $^{31}\text{P}$  NMR simultaneously with coronary blood flow and oxygen consumption in the hearts of living dogs. Three "stress tests" were used to increase cardiac work: 1) electrical pacing, 2) epinephrine and 3) phenylephrine infusions. Pacing and epinephrine caused up to 4 fold increases in cardiac oxygen consumption without significant changes in high energy phosphates. Above this level of work CrP and ATP decreased, as coronary blood flow reached maximal values, and lactate consumption of the heart decreased suggesting tissue hypoxia. In contrast, phenylephrine (which differs in that it is mainly an inotropic agent) caused changes in high energy phosphates with only a doubling of respiration. Thus, the relation between work and energy metabolism apparently depended on the way cardiac work was induced.

The NADH fluorescence on the surface of perfused rat hearts was measured, using an internal standard (chlorocarboxyfluorescein) which is trapped in the cytosol of the myocytes. The regulation of mitochondrial redox state was followed during increases in work output as well as during graded hypoxia. NADH level changed with work, apparently as a result of regulation by changes in dehydrogenase activity. This mechanism is suggested as the major link between cardiac work and metabolism in hearts under conditions in which high energy phosphate levels do not change.

The unidirectional flux rates of the creatine kinase reaction in the heart were determined in vivo using magnetization transfer techniques. The rate constant of this reaction was 0.348/sec. The rate constant was insensitive to the load of work being performed by the heart, suggesting that the reaction is at equilibrium and not involved in a unidirectional shuttling of energy between mitochondria and myofibrils.

The intracellular pH of the myocardium in vivo was 7.15, measured using the chemical shift of inorganic phosphate in the  $^{31}\text{P}$  NMR spectrum. The assignment of the inorganic phosphate resonance was made following infusion of the dogs with oxypherol to reduce the red blood cell and 2-3DPG content of the blood. This permitted the confirmation of the inorganic phosphate resonance position by removing the 2-3 DPG interference. With changes in respiration sufficient to vary arterial pH from 7.2 to 7.6 no significant changes in myocardial pH were observed. Below arterial pH of 7.2 intracellular pH fell.



NMR imaging. A new technique was developed that uses magnetization transfer to image reaction rates. It relies on the chemical exchange of nuclei after one of the nuclei is specifically labeled with an external application of radiofrequency energy. By comparing images collected with and without irradiation, the distribution of a reaction can be recorded as an image. The new technique was used to determine the distribution of the creatine kinase reaction in rabbit legs as a demonstration.

Proton NMR images were made of rabbit kidneys in vivo with 50 micron resolution. In these images structure of small blood vessels and flow through them could be resolved. The proton images were used to localize  $^{31}\text{P}$  spectroscopy of small regions of the kidney. Concentrations of glycerolphosphorylcholine, phosphoethanoamine, and betaine in the kidney cortex or medulla were measured in vivo during acute hydration. The time course of change in these "osmolytes" with acute hydration was much slower than changes in urine concentration.

Deuterium images of the cat head were obtained in animals which had drunk 20% deuterated water for 5 days. The images collected in as little as 10 minutes had high resolution and contrast. There was a 10-fold difference in T2 relaxation between the intracellular and extracellular space. This permits high contrast imaging of the intracellular versus extracellular space. Images of flow in blood vessels were obtained in three seconds that had good (1 mm) spatial resolution.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01237-09 KE

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hormonal control of transport in kidney epithelia in culture

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Joseph S. Handler Section Chief LKEM, NHLBI

Others Agnes S. Preston Chemist LKEM, NHLBI  
Chester Williams Biologist LKEM, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

## SECTION

Membrane Metabolism Section

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20982

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

2.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors C  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The transport effects and the mechanism of action of hormones and other factors are studied in epithelia formed in culture. To understand the responses better, the cell biology of the epithelia is studied as well as events specific to transport. Techniques of somatic cell genetics are applied to manipulate transporters and their regulation.

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Objectives

The purpose of this project is to gain further understanding of epithelial cell biology and the regulation of epithelial transport. In the past we have identified as well as developed several continuous cell lines that form differentiated epithelia in culture. We have studied regulation of transport by hormones and other factors. Current work is focused on the application of the techniques of somatic cell genetics to the development of new approaches to studying the regulation of epithelial transport.

Methods

See previous annual reports for methods of cell culture, assay of transport activity, and biochemical analyses.

Major Findings

We are studying three continuous kidney derived cell lines that have been shown to express the sodium-coupled phosphate transporter of the proximal tubule. All three lines grow in culture media that contain as little as one percent (0.01mM) of the normal concentration of inorganic phosphate. Growth in such a low phosphate environment may indicate that regulation of serum phosphate concentrations has evolved to the higher normal level not for renal or general cell function, but for bone formation.

Proposed Course

The foregoing interpretation will be tested by examining the ability of cells other than those that have the renal sodium-coupled phosphate transporter to grow in media with extremely low concentrations of phosphate. Phosphate transport will be examined in the renal cells that have been growing in low phosphate medium for a prolonged period to see whether the expression of phosphate transporters has been increased markedly. A small increase in transport activity has been reported for these cells after brief exposure to low concentrations of phosphate. If there is a marked increase in transport, we will search for evidence that this is the result of gene amplification. If it is, we will use appropriate techniques to clone the gene for the transporter.

Publications

Handler, J.S.: Kidney cells in culture. Kidney Internat. 30:208-215, 1986.  
Steele, R.E., Preston, A.S., Johnson, J.P., and Handler, J.S.: Porous bottom dishes for culture of polarized cells. Am. J. Physiol. 251 (Cell Physiol. 20):C136-C139, 1986.





Lang, M.A., Handler, J.S., and Gainer, H.: Neurohypophysial peptide potencies in cultured anuran epithelia (A6). Am. J. Physiol. 251 (Regulatory Integrative Comp. Physiol. 20):R77-R81, 1986.

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Yanase, M., and Handler, J.S.: Cyclic AMP stimulates chloride secretion in A6 epithelia. Amer. J. Physiol. 251: (Cell Physiol. 20): C810-C814, 1986.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01246-07 KE

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Primary and continuous culture of epithelial kidney cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Maurice Burg	Chief	LKEM, NHLBI
Other:	Shunya Uchida	Visiting Fellow	LKEM, NHLBI
	Robert Balaban	Staff Fellow	LKEM, NHLBI
	Serena Bagnasco	Visiting Associate	LKEM, NHLBI
	Jenifer Bedford	Guest Worker	LKEM, NHLBI
	Takeshi Nakanishi	Visiting Fellow	LKEM, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

## SECTION

Electrolyte Transport Section

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

5.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A continuous line of cells has been established in tissue culture from rabbit renal papillary pelvic epithelium. This and other lines of renal cells are being used for studies of their transport function and resistance to hypertonicity. The methods are being applied to establish continuous lines from other renal epithelia.

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Objectives

A long term goal of the studies is to develop continuous lines of renal epithelial cells that express differentiated function. One useful line that has resulted is GRB-PAP1 from rabbit papillary pelvic epithelium. A second goal is to find out how renal cells adapt to hyperosmolality. Bacterial, plant, and invertebrate animal cells were shown to accumulate compatible, osmotically active, organic intracellular solutes when their environment became hyperosmotic. These "osmolytes" help maintain the intracellular milieu because they do not perturb vital intracellular macromolecules, in contrast to sodium and potassium salts which in abnormally high concentrations do perturb macromolecules. Most mammalian tissues are not normally hyperosmotic and presumably do not express osmolytes. The exception is the renal inner medulla which is hyperosmotic because of the renal concentrating mechanism. In previous studies we identified the principal osmolytes in rat and rabbit inner medullas as sorbitol, inositol, glycerolphosphorylcholine, and betaine. We now are studying the mechanisms and control of their accumulation in a variety of cell lines, including GRB-PAP1.

Major findings

1) Differentiated lines of cortical collecting ducts (CCD). Uchida and Burg are attempting to start continuous lines of rabbit CCD, using the method previously developed in this laboratory and which produced the GRB-MAL1 line from medullary thick ascending limbs. Single tubules are dissected and are seeded on sheep amnions in special media. No continuous lines of CCD have resulted as yet, but epithelia are growing for as long as one year and into the third passage. The following differentiation has persisted. Like native CCD, the tissue culture epithelia have a transepithelial voltage which is inhibited by amiloride and stimulated by vasopressin. The CCD epithelia in culture also express sodium absorption, potassium secretion, and bicarbonate absorption. In addition, lactate produced by the cells is excreted only to the basolateral medium, while protons from lactic acid are excreted to the apical medium. The latter finding may point to a possible source of protons for urinary acidification. Proposed course: We will continue to watch for spontaneous transformation that might result in continuous lines and will also attempt transformation with modified SV40 virus.

2) Screening of renal cells in culture for osmolytes. We previously observed that GRB-PAP1 cells accumulated a high concentration of sorbitol, but not of other osmolytes, in hyperosmotic medium. Nakanishi, Balaban, and Burg screened other lines of cells for survival in hyperosmotic medium. MDCK, LLC-PK1, LLC-PK3, A6, and GRB-MAL1 survived in high NaCl and/or urea concentrations, but grew slowly or not at all. The surviving cells adjusted their volumes and sodium and potassium levels back towards normal and accumulated osmolytes. The osmolytes were inositol, glycerophosphorylcholine, and betaine in MDCK and the LLC-PK lines. Only sorbitol (and to a much lesser extent inositol) was accumulated by GRB-MAL1 cells. Proposed course: to investigate the mechanism and control of accumulation of osmolytes by these cells.



3) Control of sorbitol accumulation by renal medullary cells. We previously found that sorbitol accumulated by GRB-PAP1 cells in hyperosmotic medium was synthesized from glucose, catalyzed by aldose reductase (AR). Following hyperosmolality, the activity of AR increased greatly because of a large increase in the amount of enzyme protein. Uchida and Burg have now studied the nature of the stimulus that induces AR. AR activity and AR protein did not begin increasing until 12 to 24 hours after the osmolality was increased and were maximum after 3 to 4 days. Triggering of this response depended on the size of the solute molecule used to increase osmolality. Raffinose (large molecular size) was equal in effect to NaCl, causing a maximum response. Urea and glycerol (small size) did not cause any response. Mannitol and sorbitol (intermediate size) caused an intermediate response. Thus, the response depended on molecular size, which most likely was translated to osmotic effectiveness. Hyperosmotic addition of raffinose caused the cells to remain shrunken for hours with high Na and K levels, whereas urea did not cause any prolonged change in cell volume or ion concentration. Elevation of cell Na by ouabain did not trigger the response. Therefore, elevated cell K or reduced volume are the most likely signals. Proposed course: To look for second messengers and to attempt to distinguish between cell volume and K concentration as the trigger.

4) Control of sorbitol level in adapted cells. The time course for induction of AR (days) was longer than the expected time course of changes in inner medullary osmolality, following, for example, antidiuretic hormone or diuretics. Therefore, Bagnasco, Bedford, Balaban and Burg studied the effect of acute changes in osmolality on sorbitol metabolism in fully induced cells. GRB-PAP1 cells were grown in hyperosmotic medium, then the osmolality was abruptly decreased to isotonic. AR remained elevated, decreasing by half only after 7 days. Cell sorbitol level fell rapidly, however, due to efflux of sorbitol into the medium. The permeability to sorbitol was very low as long as the cells remained in hyperosmotic medium, but increased at least 200-fold following decrease in osmolality. A large fraction of the sorbitol left the cells within 15 minutes. Thus, sorbitol level in adapted cells is regulated by at least two mechanisms: 1) rapid change in sorbitol efflux and 2) slow changes in AR. Proposed course: Sorbitol dehydrogenase (SoDH) catalyzes the conversion of sorbitol to fructose. We will measure SoDH activity and fructose in the various conditions to see if this enzyme has a role in the control of sorbitol levels. Also, we will investigate whether the efflux of sorbitol represents a general increase in cell permeability or whether it is specific for sorbitol.

### Publications

Burg, M.B.: Renal handling of sodium, chloride, water, amino acids, and glucose. In Brenner, B.M., and Rector, F.C. (Ed.): The Kidney 3rd edition. Philadelphia, W.B. Saunders Co., 1986, pp. 145-175.

Bagnasco, S., Balaban, R.S., Fales, H.M., Yang, Y., and M.B. Burg: Predominant osmotically active organic solutes in rat and rabbit renal medullas. J. Biol. Chem. 261: 5872-5877, 1986.





Guggino, S.E., Guggino, W.B., Green, N. and Sacktor, B.:  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels in cultured medullary thick ascending limb cells. Am. J. Physiol. 252: C121-C127, 1987.

Guggino, S.E., Guggino, W.B., Green, N. and Sacktor, B.: Blocking agents of  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels in cultured medullary thick ascending limb cells. Am. J. Physiol. 252: C128-C137, 1987.

Balaban, R.S., and Burg, M.B.: Osmotically active organic solutes in the renal inner medulla. Kidney Inter. 31: 562-564, 1987.

Bagnasco, S.M., Uchida, S., Balaban, R.S., Kador, P.F., and Burg, M.B.: Induction of aldose reductase and sorbitol in renal inner medullary cells by elevated extracellular  $\text{NaCl}$ . Proc. Natl. Acad. Sci. 84: 1718-1720, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01266-05 KE

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of epithelial cell volume

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Kenneth R. Spring	Res. Physiologist	LKEM, NHLBI
Others:	Jeffrey L. Garvin	Guest Worker	LKEM, NHLBI
	R. Joel Lowy	Senior Staff Fellow	LKEM, NHLBI
	Arthur Siebens	Senior Staff Fellow	LKEM, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION National Heart, Lung, and Blood Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	3.2	PROFESSIONAL:	3.2	OTHER:
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CHECK APPROPRIATE BOX(ES)

- |   |  |                                      |
|---|--|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |                                      |
| <input type="checkbox"/> (a2) Interviews    |  |                                      |

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Large quantities of salt and water move across epithelial cells. These cells are able to maintain a constant volume by balancing solute entry and exit. The mechanisms for epithelial cell volume regulation are under investigation in this laboratory. Optical and microelectrode studies have been performed on the gallbladder of Necturus, on the renal cortical collecting tubule of the rabbit, the toad skin, on the renal papillary epithelium, and on semicircular canals of the inner ear.



## Objectives

Our goal is to understand the control of cellular volume in epithelia. These tissues transport large quantities of solutes and water across the cell membranes. Previous work from this laboratory has shown that the movement of water can be explained by simple osmotic gradients, and that solute movements are due to transport systems in the apical and basolateral cell membranes. These transporters can be transiently activated when the cell volume is disturbed by an osmolality change. In addition the cells exhibit slow regulatory adjustments to alterations in the rate of solute entry or exit. The mechanisms which sense the volume changes are not known although both calcium and hydrogen ions appear to be involved. The activation of the transport systems has been investigated to some extent but considerably more work needs to be done to fully characterize the changes which occur in response to perturbation of cell volume.

## Methods

Over the last 12 years we have developed a unique combination of light microscopic, video and electrophysiologic methods which have enabled us to study cell volume and intracellular ionic composition. Briefly, the preparation is visualized with a high resolution light microscope, imaged with a video camera and the images processed and stored. Simultaneous electrical measurement may be made on the cells to determine other functional parameters. Measurements of the intensity of fluorescence from living cells stained with specific indicator dyes is presently underway.

## Findings

Our recent work has centered on the factors which are involved in the activation of volume regulation by gallbladder epithelium as well as in the responses of mammalian renal tubules to hormone treatment. We have shown that the transport of Cl across frog skin is accomplished by specialized cells rich in mitochondria. Optical methods have enabled the study of the functional characteristics of the renal papillary epithelium, a tissue which has not been amenable to study by other methods. Our work on renal tubule cells has enabled the first direct measurements of the water permeability of the cell membrane of the cortical collecting tubule. Our studies of the inner ear epithelium may provide the long sought after model system required for an understanding of the mechanism of formation of endolymph.

## Proposed Course

We will continue to study renal tubule cells, renal papillary epithelium, and gallbladder by the techniques described. In addition we will apply more advanced optical and video methods to the analysis of fluorescent as well as bright field images of our preparations.



Garvin and Spring have investigated the regulation of intracellular Na and Cl in *Necturus* gallbladder by the use of ion sensitive and voltage sensitive microelectrodes. They have shown that Cl exit from the cell involves Na and  $\text{HCO}_3$  and is probably due to a basolateral cotransport system. Such a cotransporter has been identified in many epithelial cells and seems to play a significant role in both transepithelial transport and in the regulation of cell pH.

Garvin and Spring studied the site and mechanism of formation of endolymph, the fluid which fills the inner ear membranous labyrinth. This fluid is unique in its composition, being almost pure KCl in mammalian inner ear. The high K content of endolymph is an absolute requirement for hearing and balance. Nothing is known about the site or mechanism of endolymph production in any vertebrate. Garvin and Spring showed that the semicircular canals of the ear of the dogfish shark, *Squalus acanthias*, secreted endolymph at a rapid rate. They were able to demonstrate the action of various drugs on the rate of endolymph secretion and to characterize the mechanism of endolymph production. This is the first model system in which endolymph formation can be studied, and it may constitute an important break through for the field of clinical otolaryngology as well as physiology.

Larsen, Ussing and Spring have investigated the route of NaCl transport across toad skin, by using optical techniques. They found that a minority cell type, the mitochondria rich cell, is responsible for the passive movement of chloride across the skin epithelium. This cell has a voltage dependent channel for chloride which was studied optically. For the first time the transport properties of the mitochondria rich cells could be investigated. This type of cell appears in many other tissues, including kidney, urinary bladder, salivary gland, and fish gill. The information obtained will be of general use in understanding the role of this type of epithelial cell.

Lowy and Spring are developing quantitative techniques for fluorescence microscopy of living cells. They are utilizing a new illumination and detection system developed by Spring and Smith for the study of living cells stained with fluorescent indicator dyes. They are also visualizing single virus particles, the VSV virus, invading renal epithelial cells. These approaches are designed to validate in living cells previous measurements on test targets and to extend the techniques to new areas of investigation.

Siebens and Spring are studying the early events in the response of renal papillary epithelium to increases in medium osmolality. They are analyzing cell composition and enzyme activity to understand what the stimulus is for the activation of the synthetic machinery in these cells which makes large quantities of sorbitol from glucose.





Publications:

Hermansson, K., and Spring, K.R.: Potassium induced changes in cell volume of gallbladder epithelium. Pflugers Arch. 407 (suppl 2): S90-S99, 1986.

Strange, K., and Spring, K.R.: Methods for imaging renal tubule cells. Kidney International. 30: 192-200, 1986.

Strange, K., and Spring, K.R.: Absence of significant cellular dilution during ADH-stimulated water reabsorption. Science. 235: 1068-170, 1987.

Strange, K., and Spring, K.R.: Cell membrane water permeability of rabbit cortical collecting duct. J Memb Biol. 96: 27-43, 1987.

Spring, K.R., and Ussing, H.H.: The volume of mitochondria rich cells of frog skin epithelium. J Memb Biol. 92: 21-26, 1986.

Spring, K.R.: Control of epithelial cell volume. In Kruck, F. and Thirau, K. (Eds.): Endocrine Regulation of Electrolyte Balance. Heidelberg Verlag, Heidelberg, 1986, pp.4-10.

Sands, J.M., Knepper, M.A., and Spring, K.R.: Na-K-Cl cotransport in apical membrane of rabbit renal papillary surface epithelium. Am J Physiol. 251: F475-F484, 1986.

Larson, M., and Spring, K.R.: Volume regulation in epithelia. In R. Gilles, Kleinzeller, A., and Bolis, L. (Eds.): Current Topics in Membranes and Transport. (In press).

Spring, K.R., and Smith, P.D.: Illumination and detection systems for quantitative fluorescence microscopy. J Micros. (In press).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01276-03 KE

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The water permeability channel regulated by anti-diuretic hormone.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Joseph S. Handler Section Chief LKEM, NHLBI

Others: Helen Murphy	Chemist	LKEM, NHLBI
Chester Williams	Biologist	LKEM, NHLBI
Mark Willingham	Section Chief	LMB, NCI
James Wade	Assoc. Professor	Univ. Maryland
H. William Harris, Jr.	Guest Worker	LKEM, NHLBI

## COOPERATING UNITS (if any)

Laboratory of Molecular Biology, NCI  
Department of Physiology, University of Maryland School of Medicine

## LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

## SECTION

Membrane Metabolism Section

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

2.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- |   |  |                                      |
|---|--|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  | C                                    |
| <input type="checkbox"/> (a2) Interviews    |  |                                      |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The study is intended to isolate the water permeable membrane units that make the urinary plasma membrane permeable to water in response to vasopressin. Because it is well characterized and easy to work with, the toad urinary bladder is used as the source of the channels. Membrane proteins are labeled covalently with radioactive iodine to identify proteins in the plasma membrane in the presence but not in the absence of stimulation by vasopressin. Following withdrawal of vasopressin, endocytosed membrane is labeled using an intracellular labeling technique. Finally, a cell fraction enriched in endocytosed membrane is isolated and used to prepare monoclonal antibodies.

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## Objectives

The water permeability of specific portions of the mammalian kidney, as well as some other urinary tract epithelia, is dramatically increased by antidiuretic hormone (vasopressin). The increase results from the fusion of aggregophores, intracellular vesicles that are believed to contain water permeable membrane, with the urinary plasma membrane of the epithelia. The purpose of this study is to isolate and characterize the water permeable membrane units that are added to the urinary plasma membrane in response to vasopressin.

## Methods

Externally exposed proteins in the urinary plasma membrane are iodinated using glucose-oxidase/lactoperoxidase. The same enzymes are endocytosed into aggregophores when vasopressin is withdrawn in order to label proteins in aggregophores. SDS-polyacrylamide electrophoresis is used to separate proteins prior to autoradiography in order to identify labeled proteins. A density shift technique based on endocytosis of horseradish peroxidase is used to purify aggregophores. The purified aggregophores are used to prepare monoclonal antibodies. The monoclonal antibodies are screened for localization to aggregophores.

## Major Findings

The density shift technique has yielded a fraction that is rich in proteins of the same molecular weights as those identified by the glucose oxidase/lactoperoxidase radiolabeling technique. Hybridomas have been cloned that produce monoclonal antibodies that colocalize with aggregophores identified by endocytosed lucifer yellow on fluorescence microscopy.

## Proposed Course

The antibodies will be localized by immunoelectronmicroscopy to confirm their binding to aggregophores. Antibodies with confirmed localization to aggregophores will be used to isolate aggregophores and constituent water permeable membrane units. Also, the antibodies will be used to search for similar units in mammalian kidney.

## Publications

Harris, H.W., Jr., Wade, J.B., and Handler, J.S.: Fluorescent markers to study membrane retrieval in ADH treated toad urinary bladder. Am. J. Physiol. 251:C274-C284, 1986.

Harris, H.W., Jr., Wade, J.B., and Handler, J.S.: Transepithelial water flow regulates apical membrane retrieval in ADH-stimulated toad urinary bladder. J. Clin. Invest. 78:703-712, 1986.

Harris, H.W., Jr., Murphy, H.E., Willingham, M.C., and Handler, J.S.: Isolation and characterization of specialized regions of toad urinary bladder apical plasma membrane involved in the water permeability response to antidiuretic hormone. J. Membrane Biol. In press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01279-02 KE

PERIOD COVERED October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Control of Cellular Energy MetabolismPRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  
PI Robert S. Balaban

## Others

Alan Koretsky

Staff Fellow

Lawrence Katz

Medical Staff Fellow

Ronald Lynch

Guest Worker

Michael Portman

Guest Worker

Julie Swain

Senior Investigator

Cardiac Surgery NHLBI

COOPERATING UNITS (if any)

Cardiac Surgery

LAB/BRANCH Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION National Heart, Lung, and Blood Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

OTHER:

3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The control of energy metabolism within intact tissues is being investigated using a variety of techniques and tissues. The relation between the rates of energy conversion, via mitochondrial oxidative phosphorylation and glycolysis, and work output is being determined in the heart in vivo under controlled conditions. In addition, studies on the isolated perfused heart, isolated mitochondria and cultured cells are also being used to compliment the in vivo studies. In all of these preparations a tight coupling between the rate of work and the rate of energy conversion has been observed. In order to gain insight into the mechanism of this coupling, several of the key metabolic intermediates are also being determined as a function of work output using non-invasive techniques. Adenosine di- and tri- phosphates, inorganic phosphate, creatine phosphate pH and the steady state turnover of these compounds are being monitored using <sup>31</sup>P NMR. In vivo mitochondrial redox state and oxygenation is being monitored using optical spectroscopy. Classical models concerning the control of energy conversion with in cells involve the intracellular concentrations of adenosine di and tri-phosphates as well as inorganic phosphate. However, in our in vivo and perfused heart studies we have demonstrated that these compounds are not the major determinants of the rate of energy conversion. Further, in both the isolated perfused heart and mitochondria studies we have demonstrated that the redox state of NADH can control the rate of mitochondrial respiration and that the NAD redox state does change appropriately (i.e. becomes more reduced) when the isolated perfused heart is stimulated to more work. These data suggest that the control of energy conversion of the heart is actually controlled at the level of substrate oxidation most likely involving the regulation of substrate dehydrogenase activity.

576.





## Major Findings

1) We have developed an in vivo dog heart model where  $^{31}\text{P}$  NMR measurements can be made at 4.7 Tesla simultaneously with coronary blood flow and oxygen consumption. Using this model we have evaluated three protocols of increasing cardiac work (i.e. stress tests); electrical pacing, epinephrine and phenylephrine infusions. With pacing and epinephrine we have found that up to 4 fold increases in cardiac oxygen consumption can be induced without significant changes in high energy phosphates of the in vivo heart. Beyond this level decreases in CrP and ATP were observed as coronary blood flow reached its maximal values and lactate consumption of the heart decreased suggesting that tissue hypoxia developed at these higher work loads. In contrast, with the minimal inotropic effects of phenylephrine changes in high energy phosphates occurred with only doubling of respiration. Proposed course: To determine if alterations in work output over the lower work loads is associated with net reductions of mitochondrial NADH detected with optical spectroscopy. Measure dehydrogenase activity in the heart during work jumps to evaluate the activation of these enzymes by Beta adrenergic stimulation.

2) The unidirectional fluxes of the creatine kinase reaction in the in vivo heart have been determined using magnetization transfer techniques. The rate constant of this reaction is  $0.348 \text{ sec}^{-1}$ . Measuring this reaction at different work loads demonstrated that the rate constant was insensitive to the level of work being performed by the heart suggesting that the reaction is truly at equilibrium and not involved in a unidirectional shuttling of energy between the mitochondria and myofibrils. Proposed course: To determine the transmural distribution of this reaction in the in vivo heart, and examine the effects of hypoxia and beta adrenergic stimulation on this distribution.

3) The intracellular pH of the in vivo myocardium has been determined to be 7.15 using the chemical shift of inorganic phosphate in the  $^{31}\text{P}$  NMR spectrum. The assignment of the inorganic phosphate resonance was made by exchange infusion of the dogs with oxypherol to reduce the red blood cell and 2-3DPG content of the blood by 90%. This permitted the confirmation of the inorganic phosphate resonance position by removing the 2-3 DPG interference. The effects of respiratory acidosis on the myocardial pH was determined. Over the range of arterial pH of 7.2 to 7.6 no significant changes in intracellular pH were observed. Below an arterial pH of 7.2 intracellular pH does tract extracellular pH with respiratory acidosis. Proposed course: To determine the effects of intracellular pH on hypothermia recovery in experimental animals to evaluate clinical cardiac surgery procedures.

Publications:

Koretsky, A.P., and Balaban, R.S.: Changes in pyridine nucleotide levels alter oxygen consumption and extra-mitochondrial phosphates in isolated mitochondria: A  $^{31}\text{P}$  and fluorescence study. Biochem. Biophys. Acta. (In press.)

Lynch, R.L., and Balaban, R.S.: Coupling of aerobic glycolysis and Na-K-ATPase in the renal cell line MDCK. Amer. J. Physiol. (In press).

Balaban, R.S., Katz, L., and Koretsky, A.P.: NMR Investigations of Cellular Energy Metabolism. Proc. NY Acad. Sci. (In press).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01280-02 KE

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Non-invasive techniques for monitoring cellular function and structure

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI Robert S. Balaban Research Physiologist

Others	Alan Koretsky	Staff Fellow	
	Lawrence Katz	Medical Staff Fellow	
	Paul Hsieh	Research Scholar	HHMI
	Steven Wolff	Research Scholar	HHMI
	Joe Ackerman	Associate Professor	Wash. Univ.
	Colleen Ewy	Graduate Student	" "

## COOPERATING UNITS (if any)

Howard Hughes Medical Institute  
Dept. of Chemistry, Washington University, St. Louis, MO

## LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These investigations are devoted to the development of non-invasive methods of accessing cellular structure and function. Two general techniques are being used: Nuclear Magnetic Resonance (NMR) and optical spectroscopy. Over the last year we have developed a new NMR imaging technique which permits the imaging of chemical reaction rates as well as detect the presence of molecules in concentrations as low as 1 umolar due to their chemical exchange with substances in larger concentration. Different methods of determining the flux of creatine kinase in vivo have been evaluated and quantitatively compared. We have also developed a NMR microscopic technique which has permitted the to imaging of small vessels and structures in the in vivo kidney. NMR imaging and flow studies using  $^2\text{H}$  was developed and demonstrated in the in vivo environment. Using optical spectroscopy, a procedure for monitoring NADH fluorescence of the surface of the heart was developed along with the use of an trapped internal reference compound.

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## Major findings.

1) A new technique was developed using magnetization transfer to image reaction rates using NMR. This technique relies on the chemical exchange of nuclei after one of the nuclei is specifically labeled with an external application of radiofrequency energy. By comparing images collected with the irradiation and without the topological distribution of a reaction can be accessed. Using this technique we have determined the distribution of the creatine kinase reaction in the leg of the rabbit and demonstrated the utility of this technique in monitoring compounds exchanging with water. Proposed course: To use this technique to determine the distribution of creatine kinase activity in heart and brain tissue in vivo as well as investigate the use of this technique in determining the presence and turnover of metabolites in intact tissues.

2) NMR images have been obtained from the in vivo kidney with 50 micron resolution. These images demonstrate the ability to monitor the flow and structure of vessels in this size range in vivo. Using NMR spectroscopy, the concentration of organic solutes such as glycerolphosphoryl choline, phosphoethanoamine, and betaine have been monitored in the in vivo kidney during acute hydration. These studies have revealed that the time course of change in these "osmolytes" with acute hydration is very slow when compared to the change in papillary osmotic concentration. Proposed course: To use these techniques to quantitate regional renal blood flow under normal and diseased states. To image the distribution of organic solutes in the kidney, and investigate the mechanisms involved in the control of their distribution and concentration.

3)  $^2\text{H}$  images of the cat head were obtained in animals which were drinking 20%  $^2\text{H}_2\text{O}$  for 5 days before the experiments. High resolution and contrast  $^2\text{H}$  images were obtained in as little as 10 min.  $T_2$  relaxation studies reveal that there is a 10 fold difference in  $T_2$  between the intracellular and extracellular space. Preliminary studies have also demonstrated that flow images of water distribution can also be obtained with excellent time (3 sec) and spatial (1 mm) resolution. Proposed course: To use  $^2\text{H}$  to evaluate the relaxation mechanisms in biological tissues. To use  $^2\text{H}$  to determine the distribution of flow through the kidney and brain.

4) Using an internal standard which is trapped in the cytosol of the myocytes (chlorocarboxyfluorescein) the NADH fluorescence from the surface of the perfused heart has been successfully quantitated. Using this technique we have monitored the regulation of the mitochondrial redox state in the perfused heart during increases in work output as well as graded hypoxia. These data suggest that the regulation of this key metabolite by the regulation of dehydrogenase activity may play a key role in the regulation of energy metabolism.



Publications:

Balaban, R.S.: Biological Applications of NMR Spectroscopy. In Magnetic Resonance of the Reproductive System. New Jersey, Slack Inc., 1986, pp. 43-49.

Balaban, R.S.: Non-invasive approaches to the study of renal metabolism and active ion transport. In Modern Techniques of Ion Transport. New York, Churchill Livingston Inc., 1986, pp. 91-104.

Koretsky, A.P., Katz, L.A., and Balaban, R.S.: Determination of NADH Fluorescence from the perfused heart using an internal standard. Amer. J. Physiol. (In press).

Hsieh, P.S., and Balaban, R.S.:  $^{31}\text{P}$  Imaging of in vivo creatine kinase reaction rate. J. Mag. Res. (In press).





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01281-02 KE

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Renal Molecular Biology

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Arlyn Garcia-Perez Guest Worker LKEM, NHLBI

Others: Shunya Uchida	Visiting Fellow	LKEM, NHLBI
Moo Kwon	Visiting Fellow	LKEM, NHLBI
Helen Murphy	Chemist	LKEM, NHLBI
Joseph S. Handler	Section Chief	LKEM, NHLBI
Maurice B. Burg	Laboratory Chief	LKEM, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

## SECTION

Membrane Metabolism Section and Renal Mechanisms Section

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

## OTHER:

2.0

0.5

## CHECK APPROPRIATE BOX(ES)

- |   |  |                                      |
|---|--|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  | C                                    |
| <input type="checkbox"/> (a2) Interviews    |  |                                      |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The project is designed to apply the techniques of molecular biology to the kidney. Initially, the gene for aldose reductase in the kidney is being cloned and sequenced. The starting material is a continuous cell line derived from rabbit renal medulla. The cells produce increased levels of aldose reductase when grown in hypertonic media. A cDNA library prepared from cells grown in hypertonic medium will be prepared in  $\lambda$ gt11. Expression of aldose reductase will be detected using a high affinity polyclonal antibody.

581



## Objectives

Many important problems in renal physiology have reached the point where further understanding requires information about the regulation of synthesis and knowledge of the structure of proteins such as transporters and enzymes involved in metabolic pathways unique to the kidney. This project is designed to apply the techniques of molecular biology to those problems.

Evidence obtained in this laboratory indicates that renal medullary cells survive in their usual hypertonic environment because they produce intracellular osmolytes. One of the osmolytes is sorbitol which is the product of aldose reductase catalyzed reduction of glucose. We have in culture a continuous renal medullary cell line (GRB-PAR1) that expresses this enzyme when grown in a hypertonic medium. The objective of the study is to clone and sequence the gene for aldose reductase from renal medulla. The cloned gene will be used to prepare probes to study the regulation of the expression of the gene in renal medulla.

## Methods

A new  $\lambda$ gt11 library is being prepared from polyA+RNA of GRB-PAP1 cells grown in hypertonic medium for 24hr. to optimize for abundance of aldose reductase message. The amino acid composition and sequence of aldose reductase is being determined to facilitate the preparation of nucleotide probes for the gene.

## Major Findings

A screening system has been developed using polyclonal goat antiserum against aldose reductase. No true positive clone has been found among one million recombinants screened for expression using the antibody. The antibody has been affinity purified for use in future screening.

## Proposed Course

Peptide fragments of aldose reductase will be purified and sequenced. Oligonucleotide probes will be constructed based on a sequence of 5 to 6 amino acids with the least degeneracy in their expected nucleotide code. These probes will be used for screening the libraries described above.

## Publications

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01282-01 KE

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Solute and Water Transport in Renal Epithelia

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Mark A. Knepper Senior Investigator

## Others:

J.L. Garvin	Guest Worker
Jeff Sands	Senior Staff Fellow
Hiroshi Nonoguchi	Visiting Fellow
Randall Packer	[Fill in title]/ Guest Worker
Robert Star	Senior Staff Fellow
Raymond Mejia	Mathematician

## COOPERATING UNITS (if any)

M.B. Burg Chief

## LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.9

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The kidney contains several distinct epithelia that, in their aggregate function, are responsible for formation of the urine. We are studying the roles of these epithelia in the regulation of the excretion of water, urea, ammonium, bicarbonate, sodium, potassium, and chloride. The general approach is to dissect the epithelia from the kidney and to study their functions in vitro. Studies in the proximal tubule of the rabbit have demonstrated transport of both ammonium ions and ammonia molecules. Bicarbonate absorption and ammonium secretion have been demonstrated for the first time in the proximal straight tubule of the rat. Studies in the rabbit thick ascending limb have demonstrated active reabsorption of ammonium ions. Studies in rabbit cortical collecting ducts have shown formation of a disequilibrium pH which enhances ammonia secretion. Experiments in the outer medullary collecting duct have demonstrated functional carbonic anhydrase in the lumen of the inner stripe segment, but not in the outer stripe segment. Experiments in the inner medullary collecting duct have shown that vasopressin increases the urea permeability of the terminal part, but not the initial part of the inner medullary collecting duct. The increase urea permeability is mediated by a rise in intracellular cyclic AMP, but vasopressin also increases intracellular calcium. Experiments in the inner medullary collecting duct have shown that atrial natriuretic factor inhibits vasopressin-stimulated water permeability and reduces chloride permeability. Experiments in isolated rabbit papillary surface epithelium have demonstrated a low osmotic water permeability that is not affected by vasopressin.

583



## Objectives

The long term goal of this project is to understand how the kidney regulates water, urea, ammonium, bicarbonate, sodium, potassium, and chloride excretion. The approach is to study how each of these substances is transported across each of the epithelia that make up the renal tubule, and how these transport processes are regulated. The information from these studies is integrated using mathematical modelling to yield an improved understanding of how the normal kidney functions.

## Results

### A. Studies in proximal tubules.

Acid-base transport by proximal straight tubules (Garvin and Knepper). A technique was developed to dissect and perfuse proximal straight tubules from rats. Physiologically significant rates of fluid absorption, bicarbonate absorption, and ammonia secretion were measured in the isolated perfused tubules.

### B. Studies in thick ascending limbs.

Active ammonium absorption by thick ascending limbs (Garvin, Burg and Knepper). Previous studies had demonstrated absorption of ammonium ions by isolated perfused thick ascending limbs. Studies were carried out to determine whether this absorption occurs passively (driven by the lumen positive voltage), or whether ammonium is actively reabsorbed. The experiments were done in isolated perfused medullary thick ascending limbs from rabbits. When the transepithelial voltage was changed from lumen positive to lumen negative by applying NaCl gradients, the ammonium absorption persisted indicating that the ammonium absorption did not depend on the lumen positive voltage. The measured permeability to ammonium ions was  $13 \times 10^{-5}$  cm/s, a value too low to account for the measured absorption by passive processes alone. The  $\text{NH}_3$  permeability was low ( $0.3 \times 10^{-2}$  cm/s) allowing the thick ascending limb to maintain a large  $\text{NH}_3$  gradient. The results were interpreted to show that ammonium ions are actively absorbed by the thick ascending limb.

### C. Studies in cortical collecting ducts.

Disequilibrium pH and ammonia secretion by rabbit cortical collecting ducts (Star, Kurtz, Mejia, Burg, and Knepper). Isolated perfused cortical collecting ducts from rabbits were perfused with a pH-sensitive dye 1,4-DHPN to measure luminal pH. A significant acidic disequilibrium pH was found which was dissipated when carbonic anhydrase was added to the lumen. A protocol was devised to measure the effective rate constant for carbonic anhydrase dehydration in the lumen. The value was identical to the literature value indicating that there is an absolute lack of carbonic anhydrase in the lumen. The results are consistent with the proposal that a disequilibrium pH in the lumen enhances ammonia secretion in the cortical collecting duct.

### D. Studies in inner medullary collecting ducts.

Vasopressin effects on water and urea transport in inner medullary collecting duct subsegments (Sands, Nonoguchi, Knepper). Isolated perfused inner medullary collecting ducts (IMCDs) from the outer (initial IMCDs) and inner (terminal IMCDs) were studied.





Vasopressin increased both the urea permeability and the osmotic water permeability of the terminal IMCD, but only increased the osmotic water permeability of the initial IMCD. Regulation of urea permeability of the terminal IMCD is proposed to play an important role in the urinary concentrating mechanism.

Intracellular calcium and cyclic AMP as second messengers for vasopressin in the rat terminal IMCD (Star, Nonoguchi, and Knepper). In isolated perfused terminal IMCDs, vasopressin was found to increase intracellular calcium activity (using Ca-sensitive dye INDO-1) and cyclic AMP accumulation (using radioimmunoassay). Both effects occur within 1 min of exposure to vasopressin which is coincident with measured increases in urea permeability. Dose-response studies show that cAMP accumulation and urea permeability increases occur at about the same vasopressin concentration, but that a rise in intracellular calcium requires a much higher concentration of vasopressin. Addition of 8-bromo-cAMP increased urea permeability without affecting intracellular calcium. These results are consistent with the conclusion that cyclic AMP mediates the vasopressin-induced increase in urea permeability. The second messenger role of calcium and the phosphoinositide pathway (if any) remain to be discovered.

Atrial natriuretic factor (ANF) effects on water and solute permeabilities of the rat terminal IMCD (Nonoguchi, Sands, and Knepper). ANF binds to inner medullary collecting ducts and causes large increases in cyclic GMP production by activating a membrane bound guanylate cyclase. A physiologic effect of ANF on water and/or solute permeability has been proposed. We measured the effect of ANF on osmotic water permeability, urea permeability, chloride permeability, and sodium permeability of isolated perfused inner medullary collecting ducts. ANF decreased osmotic water permeability by almost 50% when the tubules were exposed to submaximally stimulating concentrations of vasopressin (0.1 nM) throughout the experiments. ANF had no effect on osmotic water permeability when vasopressin was absent or when vasopressin was present in supramaximal stimulating concentrations (10 nM). ANF decreased the apparent chloride permeability of the epithelium by about 20%. ANF had no effect on urea permeability or apparent sodium permeability. ANF had no effect on cyclic AMP accumulation by isolated IMCDs either in the presence or absence of the phosphodiesterase inhibitor IBMX. While the demonstrated effects on water and chloride permeability may play some role in the natriuresis induced by ANF, such a conclusion must await formal mathematical modelling for a quantitative evaluation.

E. Studies in the papillary surface epithelium (PSE).

Water and electrolyte transport across isolated PSE (Packer, Sands, and Knepper). Studies are in progress that will determine the passive permeability properties of the rabbit PSE utilizing an in vitro preparation mounted in an Ussing chamber. Thus, far we have found that the osmotic water permeability and urea permeability are low and uninfluenced by vasopressin. The chloride and sodium permeabilities are higher than predicted from transepithelial resistance measurements, raising the possibility of an electroneutral transcellular permeation pathway.



Annual Report  
Laboratory of Molecular Cardiology  
National Heart, Lung, and Blood Institute  
October 1, 1986 through September 30, 1987

The Laboratory of Molecular Cardiology is studying the regulation of contractile proteins in smooth muscle and non muscle cells. We are also cloning the cDNA for two proteins, myosin (heavy chain) and myosin light chain kinase so that we can study the regulation of the expression of these proteins and gain insight into their function, in nonmuscle cells. The role of DNA methylation in regulating the expression of tropomyosin is also under investigation.

The major purpose of the laboratory is to understand regulatory mechanisms. On the protein level we are seeking to understand the role of myosin phosphorylation in regulating contractile activity. Although our early studies centered on the phosphorylation of the 20,000-dalton light chain of myosin by myosin light chain kinase, we are now focusing on other kinases such as protein kinase C and other substrates (in addition to the 20,000-dalton light chain) such as the myosin heavy chain.

On the nucleic acid level, we are seeking to clone two cDNAs which we believe will give us important insights into basic cellular mechanisms such as cell motility, cell secretion and cytokinesis. The cDNAs under study are from a human lymphocyte library. One cDNA codes for human myosin heavy chain and the other for myosin light chain kinase. There were a number of reasons for choosing a human lymphocyte library. First, our laboratory where possible, would like to focus on human biology. Second, we have on hand a large supply of polyclonal antibodies made to human platelet myosin which crossreact with human platelet lymphocyte myosin, and therefore can be used to screen a lambda gt11 (expression) library. In addition, we are studying the phosphorylation of human myosin and have isolated sufficient quantities of the platelet myosin heavy chain for amino acid sequencing. This has allowed us to construct oligonucleotides to use as probes for screening a lambda gt10 library. A similar approach, (the use of affinity purified polyclonal antibodies as well as oligonucleotides) is being used to clone the cDNA for the calmodulin-dependent enzyme, myosin light chain kinase.

In addition to our new program in molecular genetics we continue to study the role of phosphorylation in regulating contractile activity in smooth muscle and nonmuscle cells. As outlined below, we are carrying out studies using strips of tracheal smooth muscle, cultured basophil and aorta cells, in vitro motility assays and purified proteins. We are seeking, for example, to learn whether myosin phosphorylation is causally related to granular secretion in nonmuscle cells, such as basophils.

One aim of this laboratory is to eventually combine the protein and molecular genetic research programs. An example of how this might occur can be illustrated as follows: One of the first steps in the onset of atherosclerosis is the proliferation of the smooth muscle cells in the aorta. We are interested in the mechanism by which a smooth muscle cell, which usually responds to an environmental stimulus by contracting, suddenly or gradually responds by proliferating. It is not unreasonable to postulate that both myosin phosphorylation and myosin gene regulation may play a causative



role in this altered response, along of course, with other factors. The ability to study mechanisms that alter contractile protein activity on a biological level and contractile protein gene expression on a molecular level, will eventually help us to understand complex diseases such as atherosclerosis.

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction (J. R. Sellers, Z01 HL 01786-08 MC). The mechanism by which tropomyosin potentiates the actin-activated MgATPase activity of phosphorylated smooth muscle myosin was investigated. Tropomyosin was found to increase the Vmax of the actin-activated MgATPase activity of smooth muscle heavy meromyosin, (HMM) but to have no effect on the Km. Tropomyosin had no effect on the actin-activated MgATPase activity of unphosphorylated HMM. Using the Nitella-based in vitro motility system and myosin coated beads the rate of movement for the following phosphorylated myosins was determined: human platelet, 0.05  $\mu\text{m/s}$ ; bovine trachea, 0.075  $\mu\text{m/s}$  and turkey gizzard, 0.25  $\mu\text{m/s}$  at 25 degrees. Each of these myosins appears to require phosphorylation for bead movement.

Platelet heavy meromyosin was prepared and phosphorylation was found to have a more profound effect on the Vmax of the actin-activated MgATPase activity than on the Km of phosphorylated and unphosphorylated myosin for actin. This is qualitatively similar to previous findings with turkey gizzard HMM.

In experiments carried out with Dr. Vladimir Shirinsky (National Cardiology Research Center, USSR) the calmodulin and actin binding protein caldesmon was shown to inhibit the movement of both smooth and skeletal muscle myosin-coated beads in the in vitro Nitella-based motility assay. The addition of tropomyosin decreased the amount of caldesmon needed for inhibition of motility, and the inhibition could be reversed by adding calcium and calmodulin.

Myosin Phosphorylation in Nonmuscle Cells (A. R. Bengur Z01 HL 91785-08 MC) In contrast to previous reports protein kinase C was found to phosphorylate the 20,000-dalton light of turkey gizzard myosin and human platelet myosin on two different sites. These sites have been identified as either serine-1 or 2 and threonine-9 in smooth muscle myosin. When intact human platelets are stimulated with phorbol esters, only the serine site is phosphorylated, suggesting that this site might be an important physiologic site for protein kinase C phosphorylation in vivo. In an in vitro assay, protein kinase C was found to phosphorylate the heavy chain of myosin in the LMM portion of the molecule. Two-dimensional peptide mapping of the myosin heavy chain yielded a single major phosphoserine peptide, suggesting that this phosphorylation was of a specific site in the heavy chain.

Phosphorylation as a Regulatory Mechanism (M. A. Corson Z01 HL 04202-06 MC) In collaboration with Dr. M. Schoenberg (NIAMS), we have initiated studies using strips of bovine trachea smooth muscle. The purpose of this study is to correlate the contractile response to certain agonists (phorbol esters, growth factors, etc.) with the state of myosin phosphorylation. Studies are underway



to see if a particular physiological response can be correlated with the phosphorylation of a specific site in the 20,000-dalton light chain of myosin and/or the 200,000-dalton heavy chain.

Myosin Phosphorylation and Basophil Secretion (I. Peleg Z01 HL 0409-01 MC) In collaboration with Drs. R. Ludowycke and M. Beaven (NHLBI)) we have been studying the time course of histamine secretion as well as myosin phosphorylation using rat leukemic basophils grown in culture. We have found that unstimulated basophils grown in culture have significant amounts of phosphate incorporated in the site phosphorylated by myosin light chain kinase. Following stimulation, which results in the secretion of histamine granules, there is de novo phosphorylation of the sites known to be phosphorylated by protein kinase C in vitro in both the light chain and heavy chain of myosin. Present studies are being conducted to separate the role of each of these sites, in the secretory process.

Growth and Differentiation of Smooth Muscle Cells (S. Kawamoto, Z01 HL 01665-12 MC) Using smooth muscle cells grown in culture we have found that both the heavy chains and light chains of myosin are phosphorylated. Moreover, myosin heavy chain phosphorylation was also observed in intact rat aorta. We believe that this is the first time heavy chain phosphorylation has been reported in mammalian smooth muscle myosin and present studies are designed to understand its significance. Treatment of cultured cells with dibutyryl cAMP resulted in a decrease in myosin light chain phosphorylation, which is in excess of 1 mole/mole in cultured cells and correlated with a change in cell shape. Interestingly, the high state of myosin light chain phosphorylation in cultured aorta cells appears to be confined to the two sites phosphorylated by myosin light chain kinase in this molecule, and not protein kinase C.

Cloning of the Gene for Mammalian Myosin Light Chain Kinase (M.A. Conti Z01 HL 04205-05 MC) The enzyme myosin light chain kinase plays an important role in regulating contractile activity in smooth muscle and nonmuscle cells. We are attempting to clone the cDNA for this enzyme and subsequently the gene. We are using both lambda gt10 and gt11 libraries and screening them with oligonucleotide probes constructed from known segments of amino acid sequence from gizzard myosin kinase and antibodies raised against gizzard myosin light chain kinase. Using these two approaches we have identified a number of putative cDNA clones and are now in the process of characterizing them.

Cloning of the Gene for Nonmuscle Myosin Heavy Chain (R. Shohet Z01 HL 04208-01 MC) We are screening a lambda gt10 and lambda gt11 library made from human lymphocyte mRNA. For lambda gt10 screening we are using oligonucleotide probes based on amino acid sequences determined from the light meromyosin fragment of human platelet myosin. For lambda gt11 screening we are using antibodies raised to human platelet myosin. Several clones have been identified in both libraries and are presently being sequenced.

Regulation of Genes for Contractile Proteins in Muscle and Nonmuscle Cells (L. Weir Z01 HL 04207-02 MC) Previous work has implicated a role for site specific demethylation in the promoter region of the gene for alpha-actin in the expression of this contractile protein. In the experiments being carried out in this laboratory a chimeric gene was constructed in which the promoter region of the rat tropomyosin I gene was placed 5' to the gene for





chloramphenicol acetyltransferase. By transfecting methylated, as well as unmethylated, constructs of this gene into fibroblasts and into myoblasts before and after induction, we plan to study the putative role of site specific demethylation in regulating CAT expression (and by inference tropomyosin I expression) in these cells. The pattern of methylation of the transfected DNA, following both transient and stable transfections, will be compared to that found for the endogenous gene in differentiated muscle cells.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01665-12 MC

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Growth and Differentiation of Smooth Muscle Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Sachiyo Kawamoto, M.D., Ph.D., Visiting Associate, LMC, NHLBI  
Robert S. Adelstein, M.D., Chief, LMC, NHLBI  
William A. Anderson, Jr., Chemist, LMC, NHLBI

## COOPERATING UNITS (if any)

Vladimir Shirinsky, Ph.D., National Cardiology Research Center, Academy of  
Medical Sciences, USSR

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## TOTAL MAN-YEARS:

1.4

## PROFESSIONAL:

1.2

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Phosphorylation and dephosphorylation of proteins are thought to be one mechanism for regulating various cell functions, including cell growth. We have been studying the potential role that phosphorylation of myosin heavy chains (MHCs) and/or myosin light chains (MLCs) may play in growth and differentiation of smooth muscle cells, using the primary culture cells derived from rat aorta. MHCs from smooth muscle myosin were found to be phosphorylated at serine and threonine residues. The stoichiometry of phosphorylation is approximately 1 mole of phosphate/mole of MHC. Phosphorylation of MHC were also observed in intact rat aorta, preferentially at serine residues. MLCs were also phosphorylated in cultured smooth muscle cells, in the presence or absence of mitogens. Myosin light chain kinase is responsible for this phosphorylation and gives two different forms of phosphorylated MLC: one is singly phosphorylated (at serine residue) and the other is doubly phosphorylated (at serine and threonine residues). Treatment of the cells with dibutyryl cAMP caused a decrease in the level of MLC phosphorylation, especially of the doubly phosphorylated form, concomitant with cellular shape change. The physiological meaning of these phosphorylations is under study.

We have been investigating the effects of some growth factors and vasoactive factors on the expression of myosin heavy chain isoforms at the protein level. Insulin, platelet-derived growth factor as well as fetal calf serum caused an increase of synthesis ( $^{35}\text{S}$ -methionine incorporation) of nonmuscle myosin. On the other hand, platelet poor plasma from adult human sources caused an increase of synthesis of smooth muscle myosin in primary culture of rat aorta.

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Project Description:

Objectives: This study is aimed at characterizing the contractile proteins, myosin and myosin light chain kinase, found in smooth muscle cells which have been grown in various culture conditions as well as those in intact tissues, and eventually at examining the underlying regulatory mechanisms of expression of the smooth muscle-specific forms of these proteins.

Methods: Primary culture and subculture of smooth muscle cells prepared from rat and rabbit aorta; immunoblots and immunoprecipitation; labeling cells with  $^{32}\text{P}$ -orthophosphate or  $^{35}\text{S}$ -methionine, 2-dimensional gel electrophoresis, 2-dimensional peptide mapping, phosphoamino acid analysis.

Major Findings: Myosin heavy chain (MHC) of smooth muscle myosin was found to be phosphorylated in primary cultures derived from rat aorta smooth muscle cells. Tryptic phosphopeptide maps showed two major phosphopeptides; one containing phosphoserine and the other containing both phosphothreonine and phosphoserine. Stoichiometry of MHC phosphorylation was estimated at near to 1 mole of phosphate per mole of MHC. MHC phosphorylation was also found in intact rat aorta, preferentially at serine residues. Phosphorylation of myosin light chain (MLC) has also been observed in cultured smooth muscle cells, in the presence or absence of mitogens. Based on the data from tryptic phosphopeptide mapping, phosphoamino acid analysis and 2-dimensional gel electrophoresis, two different phosphorylated forms of MLC were present. One is singly phosphorylated at a serine residue and the other is doubly phosphorylated at serine and threonine residues. Both forms are produced by the phosphorylation catalyzed by myosin light chain kinase. When the cells were exposed to dibutyryl cAMP, the level of MLC phosphorylation was decreased, especially at phosphothreonine residue and resulted in shape changes in the cells.

The effects of some growth factors and vasoactive agents on the expression of MHC isoforms have been studied at the protein level, using primary culture of rat aorta smooth muscle cells. Synthesis (incorporation of  $^{35}\text{S}$ -methionine) of nonmuscle MHC was increased by insulin, platelet-derived growth factor as well as fetal calf serum. Synthesis of smooth muscle MHC was increased by platelet poor plasma prepared from human adults.

In collaboration with Dr. Vladimir Shirinsky, from the National Cardiology Research Center, Academy of Medical Sciences of the USSR, preliminary studies have demonstrated that the cultured smooth muscle cells from rabbit aorta contained a MHC which was not reactive with antibodies against smooth muscle myosin nor with antibodies against nonmuscle myosin, using immunoblots. This unique MHC migrated faster than the MHCs of smooth muscle myosin found in adult rabbits on SDS-polyacrylamide gel electrophoresis.

Significance to Biomedical Research: The studies on the mechanism regulating the transitional expression of isoforms between smooth muscle and nonmuscle myosin and/or myosin light chain kinase in an in vitro system should provide useful information for understanding the events leading to the development of normal smooth muscle tissue, such as a functioning vascular wall, and to an understanding of the mechanisms by which quiescent differentiated smooth



muscle cells can be triggered to dedifferentiate and proliferate. The latter process has been implicated in some pathological states such as atheromatous plaque formation.

Proposed Course: We are seeking to uncover the factors which can change the phosphorylation levels of myosin heavy chain (MHC) and myosin light chain (MLC) in vivo, and the protein kinases which can catalyze the MHC phosphorylation in vitro, in order to understand the physiological meaning of these phosphorylations. We also plan to characterize the MHCs of smooth muscle from fetal or newborn aorta as well as those from rabbit aorta smooth muscle cells grown in culture, using peptide mapping and antibodies.

Publications:

Kawamoto, S. and Adelstein, R.S.: Characterization of myosin heavy chains in cultured aorta smooth muscle cells. A comparative study. J. Biol. Chem. 262: 7282-7288, 1987.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01785-08 MC

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Myosin Phosphorylation in Nonmuscle Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

A. Resai Bengur, M.D., Guest Researcher, LMC, NHLBI, Started 7/85  
James R. Sellers, Ph.D., Research Biologist, LMC, NHLBI  
William A. Anderson, Jr., Chemist, LMC, NHLBI  
Estelle V. Harvey, Biologist, LMC, NHLBI

## COOPERATING UNITS (if any)

Dr. Ettore Apella, NCI  
Elizabeth Robinson, NCI

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## TOTAL MAN-YEARS:

1.6

## PROFESSIONAL:

1.2

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have been investigating the phosphorylation of smooth muscle and nonmuscle myosin by protein kinase C (PKC). Protein kinase C phosphorylates the smooth muscle myosin light chain at two sites; serine-1 or 2 and threonine-9. Threonine-9 is preferentially phosphorylated at a rate approximately 2 - 3 times faster than that of serine-1 or 2. Two-dimensional tryptic peptide mapping of the 20kDa light chain of platelet myosin phosphorylated by PKC demonstrates two phosphopeptides that are similar to those seen following digestion of the smooth muscle myosin light chain. When intact human platelets are stimulated with phorbol myristate acetate, however, there is only one major phosphopeptide seen on two-dimensional tryptic peptide mapping. This corresponds to the serine site seen in the peptide map of the in vitro phosphorylated light chain. In vitro studies indicate that this in vivo specificity is dependent on ionic strength.

In addition, we have demonstrated that protein kinase C phosphorylates the myosin heavy chain in vitro, using human platelet myosin. Heavy chain phosphorylation results in a single major tryptic phosphopeptide that contains phosphoserine and has been localized to the LMM portion of the heavy chain by limited chymotryptic digestions.

To expedite two-dimensional peptide mapping of the phosphorylated light chain from in vivo systems, we have recently started to map peptides of the phosphorylated light chain using HPLC.

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Project Description:

Objectives: To determine the role of protein kinase C phosphorylation in the regulation of both smooth muscle and non-muscle myosins.

Major Findings: The sequence of the sites phosphorylated by protein kinase C in the smooth muscle myosin light chain: We have determined the sites phosphorylated by PKC in the smooth muscle myosin light chain from turkey gizzard. On two-dimensional tryptic peptide mapping of the phosphorylated light chain, it was clear that there are two major sites phosphorylated by PKC; one containing serine and the other threonine. We were able to purify and sequence these peptides by first succinylating the phosphorylated light chain and then digesting with trypsin. The tryptic peptides were purified by reverse phase and DEAE ion exchange HPLC. The sequence of the threonine containing peptide was ALA-LYS-ALA-THR-THR-LYS-LYS-ARG-PRO-GLN- where the first threonine residue is the ninth residue in from the N-terminus. By serially evaluating the products of Edman degradation, we were able to determine that threonine 9 is the site phosphorylated. The serine containing peptide is the N-terminal peptide Ac-SER-SER-CYS-ARG- as determined from the amino acid composition. As the N-terminus is acetylated we could not determine whether serine-1 or 2 is the actual site.

In vitro and in vivo phosphorylation of the platelet myosin light chain: Two-dimensional tryptic peptide maps of platelet myosin light chain phosphorylated by PKC show two major phosphopeptides that correspond to those seen in the maps of the smooth muscle light chain. When one looks however at two-dimensional tryptic peptide maps of the light chain isolated from intact human platelets stimulated with phorbol myristate acetate (PMA) phosphorylation occurs primarily at the serine site with little or no phosphorylation at the threonine site. This pattern of phosphorylation can be duplicated in vitro by increasing the ionic strength to 300 mM KCl.

Heavy chain phosphorylation of purified platelet myosin by protein kinase C: We have demonstrated that PKC can phosphorylate the heavy chain of platelet myosin in vitro. The stoichiometry of this phosphorylation is between 0.3-0.5 mol Pi/mol MHC as determined by scanning of 12.5% acrylamide SDS gels and comparing the amount of phosphorylation in the heavy chain to the light chain. This phosphorylation occurs at a serine residue that yields one major phosphopeptide on two-dimensional tryptic peptide mapping. This site has been localized to the LMM portion of the heavy chain by limited chymotryptic digestion.

Peptide mapping of the phosphorylated light chains using HPLC: We have started to map tryptic digests of the phosphorylated light chain on HPLC. Using a reverse phase column with an acetonitrile gradient we are able to successfully separate the peptide containing serine-19 which is the site for myosin light chain kinase phosphorylation, from the peptides containing serine-1 or 2 and threonine-9, the sites phosphorylated by PKC. In this system, however, we are unable to separate the two peptides containing the sites phosphorylated by PKC. On tryptic digestion, these two peptides are potentially very similar (AC-SER-SER-LYS-ARG- vs. -THR-THR-LYS-LYS-). Attempts at a DEAE ion exchange HPLC column have not been successful.



Proposed Course: We plan to study the functional significance of platelet myosin phosphorylation at a single site, serine-1 or 2, in the light chain as well as the heavy chain. This will be studied by looking at MgATPase activities, motility in the in vitro motility assay now available in the lab, and by looking at filament formation.

In addition, we plan to study heavy chain phosphorylation in more depth by trying to determine whether it occurs in vivo.

We will continue to develop a system for phosphopeptide mapping by HPLC. This will focus on varying the acetonitrile gradient as well as using other proteases for generating peptides such as the endoproteinase Arg-C.

Publications:

Bengur, A.R., Robinson, E., Appella, E. and Sellers, J.R.:  
Sequence of the sites phosphorylated by protein kinase C in the smooth muscle myosin light chain. J. Biol. Chem. 262: 7613-7617, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01786-08 MC

## PERIOD COVERED

October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

James R. Sellers, Ph.D., Research Biologist, LMC, NHLBI

Estelle V. Harvey, Biologist, LMC, NHLBI

William A. Anderson, Jr., Chemist, LMC, NHLBI

## COOPERATING UNITS (if any)

Vladimir Shirinsky, Ph.D., National Cardiology Research Center, Academy of  
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## TOTAL MAN-YEARS:

1.8

## PROFESSIONAL:

0.8

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mechanism of the phosphorylation-dependent myosin-linked regulation of smooth muscle and nonmuscle myosins is being investigated. This involves several approaches: (1) measurement of rate constants for various steps in the kinetic cycle for the hydrolysis of MgATP by heavy meromyosin (HMM), the proteolytic subfragment of myosin, in the presence and absence of actin; (2) preparation of HMM from cytoplasmic myosins and characterization of their kinetic properties, and (3) use of an in vitro motility system to quantitate how the velocity of movement of myosin-coated beads is affected by various factors. We are also studying the mechanism of action of caldesmon, a possible regulatory protein associated with actin filaments.

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Project Description

Objectives: The objective of this project is to understand how phosphorylation regulates the actin-activated MgATPase activity of smooth and nonmuscle myosins in vitro. This should help to elucidate the mechanism of the regulation of smooth muscle and nonmuscle contractile processes in vivo. To attack this problem we study how phosphorylation affects the interaction of smooth muscle and nonmuscle myosin (or their subfragments) with actin. To do this we use various enzymatic and physical techniques.

Major Findings: Kinetic Studies of the mechanism of the acto-HMM MgATPase activity: Phosphorylation of myosin by myosin light chain kinase (MLCK) is required for contraction of smooth muscle. Previous studies from this lab as well as others have shown that phosphorylation is also required for the activation of the MgATPase activity of turkey gizzard smooth muscle myosin by actin. In other words the MgATPase activity of unphosphorylated turkey gizzard smooth muscle myosin is not activated by actin. Tropomyosin is also a component of thin filaments in smooth muscle and has been shown to potentiate the actin-activated MgATPase activity of smooth muscle myosin in vitro. In order to elucidate the mechanism of this potentiation we have prepared heavy meromyosin (HMM), the soluble enzymatically active fragment of myosin. Steady state studies of the actin-activated MgATPase activity of phosphorylated smooth muscle HMM showed that tropomyosin addition to actin resulted in an increase in  $V_{max}$  with no effect on the  $K_m$ . It is known from studies by L. Greene and coworkers (NHLBI) that tropomyosin can occupy so called weak and strong positions on the actin filament where the weak position inhibits and the strong position activates the MgATPase of skeletal muscle subfragment-1 (S-1). They found that S-1 which had been extensively modified by N-ethyl maleimide (NEM S-1) could bind to actin in an ATP insensitive manner. If NEM S-1 is added to tropomyosin-containing actin filaments at ratios of 4:14 (NEM S-1:actin monomer), it pushes the skeletal muscle tropomyosin from a weak position to a strong position. We have found that actin filaments complexed with smooth muscle tropomyosin activates the MgATPase activity of phosphorylated smooth muscle HMM to an intermediate extent between the MgATPase activity found for actin alone and that found for the actin-tropomyosin-NEM S-1 system described above. In a typical experiment we find that tropomyosin increases the actin-activated MgATPase activity of phosphorylated HMM about 4 fold over that in the presence of actin alone. However if NEM S-1 is also added the MgATPase activity is further increased by a factor of two over that in the presence of actin-tropomyosin. This indicates that smooth muscle tropomyosin probably occupies preferentially the strong position and that the equilibrium can be further shifted to the strong position by the addition of attached cross-bridges.

When the same experiments are performed with unphosphorylated smooth muscle HMM we found that there was no significant activation of the MgATPase activity by either tropomyosin-actin or tropomyosin-actin + NEM S-1. This is important since it indicates that the regulation of smooth muscle HMM is not lost when the actin filaments are fully activated.

Analysis of the Motility of Myosin-Coated Beads in an in Vitro System. We have been studying the movement of myosin-coated beads in the Nitella based in



vitro motility sytem. Since smooth and nonmuscle myosins move at a slow rate in this system we have tried to find optimal ionic conditions for the maximum rate of movement. For turkey gizzard smooth muscle myosin this corresponds to a free  $Mg^{++}$  concentration of 4 mM. Our experiments indicate that under all ionic conditions tested that the movement of turkey gizzard myosin is dependent upon phosphorylation of the 20,000 kDa light chain. The maximum rate of movement of phosphorylated myosin from human platelet, bovine trachea, and turkey gizzard is 0.05, 0.075, and 0.24  $\mu m/s$  respectively at 25 degrees C. This compares to a rate of 1.85  $\mu m/s$  for rabbit skeletal muscle myosin when measured under the same conditions. Preliminary studies indicates little or no effect on the bead velocity following phosphorylation of myosin by protein kinase C or by phosphorylation of the second site of myosin light chain by myosin light chain kinase.

Regulation of Nonmuscle Myosins and Characterization of Their Enzymatically Active Subfragments: In order to characterize the mode of regulation of nonmuscle myosin from human platelets we have examined the dependence of the MgATPase activity on actin concentration. The results of five separate preparations indicated a  $V_{max}$  of 0.525/s for phosphorylated myosin and 0.046/s for unphosphorylated myosin. The  $K_m$  for phosphorylated myosin was 9.4  $\mu M$  while that for unphosphorylated myosin was 34.4  $\mu M$ . This indicates that the regulation is similar to that described for gizzard myosin. Further evidence for this stems from the observation that the movement of platelet myosin-coated beads is dependent on phosphorylation of the myosin. It is possible that the failure to observe movement with unphosphorylated myosin-coated beads could be due to lack of myosin filaments binding to the beads under these conditions. Experiments were performed that suggests that this possibility unlikely.

Mechanism of caldesmon action: Caldesmon is a 130,000 Da protein that binds to actin filaments in smooth muscle. It has been shown to inhibit the MgATPase activity of both smooth and skeletal muscle myosins. This inhibition could be reversed in the presence of calmodulin and calcium. In collaboration with Dr. Vladimer Shirinsky, National Cardiology Research Center, Academy of Medical Sciences of the USSR, we have now shown that caldesmon can inhibit the movement of both smooth and skeletal muscle myosin-coated beads in the in vitro motility assay. The concentration dependence of this inhibition was very steep. From the mid-point of the inhibition curve a disassociation constant of caldesmon to actin-tropomyosin of 42nM was determined. This value corresponds well to the values determined by other means. We found that while tropomyosin was not necessary for the inhibition it greatly reduced the amount of caldesmon required. The inhibition of bead movement could be fully reversed by the addition of calcium and calmodulin. Immunofluorescence localization studies using anti-caldesmon antibodies showed that the added caldesmon bound specifically to the actin bundles present in the Nitella preparation.

We have also examined the mechanism of caldesmon inhibition in an experiment similar to that described in section 1 above. Here we found that the presence of NEM S-1 could not reverse the caldesmon induced inhibition of the acto-HMM



ATPase activity indicating that either caldesmon does not act like troponin from skeletal muscle or that it is more potent in fixing the tropomyosin in the weak state.

A third study with caldesmon examined whether it was inhibiting ADP release from an actomyosin.ADP step in the kinetic cycle. Experiments with Dr. David Hathaway, University of Indiana School of Medicine, indicates that caldesmon has no effect on this rate.

Finally we determined that caldesmon prevents the binding of myosin light chain kinase to actin, probably by direct competition. Experiments in collaboration with Dr. Shirinsky showed that in primary rabbit aorta cultured cells the localization of myosin light kinase closely paralleled that of myosin and not that of actin. These experiments indicate that myosin and not actin is probably the intracellular binding site for myosin light chain kinase.

#### Publications:

Greene, L.E. and Sellers, J.R.: Effect of Phosphorylation on the Binding of Smooth Muscle Heavy Meromyosin·ADP to Actin. J. Biol. Chem. 262, 4177-4181, 1987.

Sellers, J.R. and Adelstein, R.S.: Regulation of Contractile Activity. The Enzymes 18, 381-418, 1987.

Adelstein, R.S. and Sellers, J.R.: Effects of Calcium on Vascular Smooth Muscle Contraction. Am. J. Cardiol. 59, 4B-10B, 1987.

Lash, J.A., Sellers, J.R. and Hathaway, D.R.: The effects of caldesmon on smooth muscle acto-HMM ATPase activity and binding of HMM to actin. J. Biol. Chem. 261, 16155-16160, 1986.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04202-06 MC

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Phosphorylation as a Regulatory Mechanism

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Marshall A. Corson, M.D., Medical Staff Fellow, LMC, NHLBI

Robert S. Adelstein, M.D., Chief, LMC, NHLBI

William A. Anderson, Jr., Chemist, LMC, NHLBI

## COOPERATING UNITS (if any)

Mark Schoenberg, M.D., LPB, NIAMS

## LAB/BRANCH

Laboratory of Molecular Cardiology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.4

## PROFESSIONAL:

1.2

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

A system for studying basic biochemical and physiologic aspects of the contraction of intact mammalian smooth muscle has been established in this laboratory during the past year. Utilizing fresh strips of bovine trachealis muscle, methods have been developed to correlate the contractile response to certain agonists with the state of myosin phosphorylation. Of particular interest is whether phosphorylation of the 20 kDa myosin light chain (MLC) at an amino acid residue(s) other than serine 19, known (Pearson, R.B. *et al.* (1984) FEBS Lett. 168, 108-112) to be preferentially phosphorylated by myosin light chain kinase (MLCK), can be shown to occur and to have a regulatory role in tension generation. Baseline studies have demonstrated the expected single phosphopeptide upon activation of MLCK via the muscarinic cholinergic receptor agonist carbachol. In contrast, upon stimulation with a phorbol ester which preferentially activates intracellular protein kinase C (PKC), preliminary results indicate the presence of an additional phosphopeptide. Experiments to more precisely characterize this response are in progress.





Project Description:

Objectives: It is well agreed that development of tension in smooth muscle requires the phosphorylation of myosin light chain, but mechanisms responsible for force maintenance have not been definitively determined. During the early phase of contraction, transients in MLC phosphorylation and shortening velocity are observed prior to the development of peak tension; however as tension becomes constant, phosphorylation and velocity decline to near baseline levels. A number of hypotheses have been advanced to explain these observations, including but not limited to: development of a "latch" state with very slowly cycling actomyosin cross-bridges, a regulatory mechanism involving thin filament-associated proteins, or activation of cellular protein kinase C with phosphorylation of as yet undetermined regulatory targets. Studies have been initially directed toward exploring the latter possibility.

The global objective of establishing a system to study intact smooth muscle is to better understand contractile events in situ and to more precisely evaluate the physiologic significance of in vitro observations made by other members of this laboratory. Specific areas of focus at present include: (1) characterizing the contractile response to known activators of PKC and evaluating its possible physiologic meaning, and (2) characterizing the nature of the contractile response to certain polypeptide serum growth factors such as platelet derived growth factor (PDGF). This project is being conducted in collaboration with Dr. Mark Schoenberg, NIAMS (see Z01 AR 27001-13 LPB).

Methods: We have developed techniques for cutting small strips of bovine trachealis muscle from specimens freshly obtained at slaughter, performing physiologic measurements (such as tension generation and unloaded shortening velocity in response to chemical and electrical depolarization, as well as to soluble contractile agonists), freezing the tissue and extracting the contractile proteins. The state of MLC phosphorylation has been evaluated quantitatively using two-dimensional gel electrophoresis and urea-glycerol electrophoresis with radioimmunoblotting, and qualitatively (localization of amino acid site phosphorylated) using tryptic phosphopeptide and phosphoamino acid analysis.

Major Findings and Proposed Course: In preliminary studies, various smooth muscle strip preparations were evaluated for their overall suitability, including rabbit trachealis and aorta, canine trachealis and coronary artery in addition to bovine trachealis. The latter was chosen for a number of reasons: high cellular content of muscle strips with cells favorably aligned in parallel such that force is distributed along rather than across the long axis of the strips, low rest tension, and the amenability of this tissue to electrical field stimulation for synchronous contraction of individual cells. A protocol for conditioning freshly hung tracheal strips and determining the optimum length for tension generation was developed.

To establish a baseline by which to evaluate the contractile response to phorbol ester (activator of PKC) and growth factors, the response of bovine trachealis to the muscarinic cholinergic agonist carbachol was studied. This agonist induces a sustained contraction with half-time to maximum force development of approximately three minutes, with peak MLC phosphorylation



occurring within this same time period. Tryptic phosphopeptide analysis of MLC revealed a single phosphopeptide throughout the time studied, from one to fifteen minutes. While the amino acid phosphorylated remains to be determined by phosphoamino acid analysis, the phosphopeptide migrates similarly to that containing phosphoserine-19, phosphorylated by MLCK.

Previous attempts to stimulate tracheal contraction with various phorbol esters, reported by other investigators, have met with variable success; using the hydrophilic species phorbol-12,13 dibutyrate (PDB), we have observed a reproducible contractile response with a markedly slowed onset but similar peak tension to carbachol. Initial results of phosphopeptide analysis indicate that a second phosphopeptide appears early in the contraction time course; studies to identify this phosphoamino acid are in progress.

The polypeptide mitogens PDGF and EGF (epidermal growth factor) have been shown (Berk, B. et al. (1986) Science 232, 87-90) to be contractile agonists for vascular smooth muscle at nanomolar concentrations, at which they are believed to be mitogenic. On a molar basis PDGF appears to be the most potent vasoconstrictor substance yet identified. As myosin isoform expression appears to be related to cellular phenotype (ie contractile vs. competent for growth) it is our hope to better elucidate how these hormones regulate commitment to the contractile pathway.

#### Publications:

Tobacman, L.S.: Activation of actin cardiac myosin subfragment 1 MgATPase rate by Ca<sup>2+</sup> shows cooperativity intrinsic to the thin filament. Biochemistry 26: 492-497, 1987.

Tobacman, L.S., and Lee, R.: Isolation and functional comparison of bovine cardiac troponin T isoforms. J. Biol. Chem. 262: 4059-4064, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04205-05 MC

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cloning of the Gene for Mammalian Myosin Light Chain Kinase

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Mary Anne Conti, Ph.D., Research Chemist, LMC, NHLBI

Robert S. Adelstein, M.D., Chief, Laboratory of Molecular Cardiology, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Cardiology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.1

## PROFESSIONAL:

1.1

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have screened two cDNA libraries from human nonmuscle cells for myosin light chain kinase. Twenty-four positive clones have been identified and are being characterized with regard to size, cross-hybridization with each other, size of messenger RNA and sequence.

603



Project Description:

Objectives: The contractile proteins, actin and myosin are believed to play a major role in nonmuscle cell functions such cell division, secretion and intracellular translocation. Since activation of myosin's ATPase activity is controlled by phosphorylation, we have been interested in the regulation and expression of the enzyme responsible for this phosphorylation, myosin light chain kinase (MLCK). We are cloning the gene for MLCK in order to look at expression and regulation of this key muscle protein in a nonmuscle cell.

Methods: Nucleic acid hybridization; agarose gel electrophoresis; cDNA cloning; antibody screening of cDNA expression libraries; oligomer screening of lambda gt10 libraries; restriction analysis; affinity columns; cell culture.

Major Findings: A Jurkat T-lymphocyte library in lambda gt11 has been screened with an affinity-purified polyclonal antibody raised against avian smooth muscle MLCK. Ten positive clones have been isolated and characterized with regard to size and hybridization to Jurkat cell RNA. In addition, the Jurkat cDNA library in lambda gt10 is being screened with a synthetic oligomer which is complementary to a portion of avian smooth muscle MLCK. Fourteen initial positive clones have been identified. These will be purified and further characterized by size, hybridization to Northern blots, and cross-reactivity to the clones isolated from the lambda gt11 library. Sequence homology to the MLCK from turkey gizzard smooth muscle will be used as a final means of identification.

Proposed Course: When the cDNA clones for MLCK are sequenced and positively identified they will be used to probe genomic libraries. The location of promoter and enhancer sequences, of introns and exons will be identified in an effort to understand gene regulation and expression as well as the function of MLCK in nonmuscle cells.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04207-02 MC

PERIOD COVERED

October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Genes for Contractile Protein in Muscle and Nonmuscle Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Lawrence Weir, Ph.D., Visiting Associate, LMC, NHLBI  
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.1

PROFESSIONAL:

1.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A chimeric gene was constructed in which the promoter region of the rat tropomyosin I gene was placed 5' to the gene for bacterial chloramphenicol acetyltransferase (CAT). We intend to use this construction to study the involvement of methylation in the regulation of tropomyosin gene expression. Towards this end we have performed a number of preliminary experiments aimed at the optimization of DNA transfection using several cell types.

605



### Project Description

Objectives: We intend to determine the mechanisms by which genes may be transcriptionally regulated by methylation of DNA.

Methods: Southern blot hybridization; DNA mediated transfection; in vitro methylation of DNA; CAT assays, construction of plasmid vectors.

Major Findings: The plasmid p-alpha CAT containing the skeletal muscle alpha actin promoter 5' to the CAT gene has been transfected into mouse fibroblasts and rat myoblasts. CAT expression indicates that the gene is functional in both cell types though at a low level compared to the control plasmid pSV2CAT (which contains the SV40 early promoter). In order to study the regulation of the rat tropomyosin I gene a plasmid (p210TMCAT) was constructed in which the tropomyosin I gene is placed 5' to the CAT gene. Preliminary experiments show that this plasmid is also functional with respect to CAT expression but again at a low level. When the transfection protocol has been optimized these constructs will be used to study the effect of methylation on the expression of the various promoters. The plasmids have been methylated in vitro and will be transfected into different cell lines. Since expression of a gene is usually (but not always) correlated with demethylation it should be possible to find specific DNA sequences in the promoter which are recognized by tissue specific factors and are specifically demethylated in the process of transcriptional activation.

### Publication:

Yisraeli, J., Adelstein, R.S., Melloul, D., Nudel, V., Yaffe, D and Cedar, H.: Muscle-specific activation of a methylated chimeric actin gene. Cell 46, 409-416, 1986.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04208-01 MC

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cloning of the Gene for Nonmuscle Myosin Heavy Chain

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Ralph V. Shohet, M.D., Medical Staff Fellow, LMC, NHLBI

Robert S. Adelstein, M.D., Chief, LMC, NHLBI

Yvette A. Preston, Biologist, LMC, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Cardiology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.2

## PROFESSIONAL:

1.2

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have obtained several putative clones of myosin heavy chain from both lambda gt10 and lambda gt11 cDNA libraries of human lymphocytes. Initial attempts to establish their authenticity by sequencing and northern analysis is underway. We also intend to sequence other myosin heavy chain tryptic fragments, especially the peptide containing the protein kinase C phosphorylation site, from which additional oligonucleotide probes will be derived. Other strategies for obtaining appropriate probes and libraries may also need to be considered. When the cDNA and/or gene have been cloned we anticipate using this information to study nonmuscle myosin expression and structure.

607



Project Description:

Objectives: The elucidation of such fundamental cell processes as cytokinesis, secretion and receptor responses requires a greater understanding of contractility in nonmuscle cells. We wish to explore the structure and role of the contractile apparatus, especially myosin, in nonmuscle cells. We have reason to believe that the regulation of nonmuscle contractile genes may be intimately bound up with the changes which occur when cells are immortalized in culture. Also the specific structural characteristics of nonmuscle myosin have begun to provide insights into its role in cells (cf. DeLozanne and Spudich, Science v.236, p.1086). With both of these goals in mind, we have begun to isolate probes for mammalian nonmuscle myosin from a lymphocyte library.

Methods: Northern and Southern analysis, Sanger dideoxy sequencing, antibody and oligonucleotide screening of cDNA libraries, HPLC chromatography, phosphorylation of nonmuscle myosin.

Major Findings: Four putative clones for lymphocyte myosin heavy chain (MHC) have been isolated from a lamda gt11 library with an antibody to platelet MHC. Also four putative clones have been obtained from a lamda gt10 lymphocyte library using a degenerate oligonucleotide probe synthesized from an HPLC purified tryptic peptide of platelet MHC. These clones are being characterized using a combination of Northern analysis and DNA sequencing. None has yet been demonstrated to hybridize to a mRNA sufficiently large to code for a traditional MHC, nor has sequence revealed significant homologies with known MHCs.

Proposed Course: The characterization of the eight clones described above will be completed. If this is not successful further protein sequencing of MHC peptides will be pursued; specifically the tryptic peptide containing the protein kinase C phosphorylation site will be sequenced. Encouragingly, recent demonstration of a six kilobase message from lymphocytes hybridizing to the ATP-binding site of nematode MHC cDNA could be followed up by using this and more 3' fragments to screen both genomic and/or random primed cDNA nonmuscle libraries.

When appropriate probes have been obtained developmental studies of MHC gene expression in cultured cells will commence. Concurrently, structural analysis of the coding sequence is expected to produce useful insights into nonmuscle MHC function.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 04209-01 MC

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Myosin Phosphorylation and Basophil Secretion

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Itzhak Peleg, Ph.D., Visiting Fellow, LMC, NHLBI  
Robert S. Adelstein, M.D., Chief, LMC, NHLBI  
William A. Anderson, Jr., Chemist, LMC, NHLBI

## COOPERATING UNITS (if any)

Russell Ludowyke, Ph.D., CP, NHLBI  
Michael Beaven, Ph.D., CP, NHLBI

## LAB/BRANCH

Laboratory of Molecular Cardiology

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.4

## PROFESSIONAL:

1.2

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are investigating the role of myosin phosphorylation in causing histamine secretion from rat leukemic cells. Upon stimulation, there was a significant increase (2-3 fold) of phosphate incorporation into the myosin heavy and 20 kDa light chains of antigenically primed cells compared to non-stimulated control cells. Maximal phosphorylation was observed at a time (2.5-10 min) when the calcium signal and rate of inositol phospholipid hydrolysis have been shown to be maximal. Two dimensional tryptic peptide maps indicated that the light chains were phosphorylated by myosin light chain kinase (serine -19 site) and protein kinase C (serine -1 or serine -2 site). Whether this phosphorylation of myosin within intact basophils plays a role in the secretory process is under investigation.



Project Description:

Objectives: To establish the possible relationship of a change in myosin phosphorylation with histamine secretion from rat basophil leukemic cells. This will be approached by following the time-course of phosphorylation of the 20,000-dalton light chains of myosin and the myosin heavy chains by precipitating these peptides with an antibody raised against platelet myosin. The various phosphorylated sites as well as the stoichiometry of the phosphorylation will be determined along with the amount of histamine released from stimulated and control cells. This work is being conducted in collaboration with Dr. Russell Ludowycke and Dr. Michael Beaven, NHLBI LCP (See Z01 HL 00993-01 CP).

Methods: Immunoprecipitation of myosin using anti-platelet myosin antibodies; 2-dimensional peptide mapping; electrophoresis and chromatography or, isoelectric focusing and electrophoresis; phosphoamino acid analysis.

Major findings: When IgE is attached to receptors present on the surface of the rat basophil leukemic cell line (RBL2H3) and cross-linked by a specific antigen (DNP-BSA), there is a breakdown of polyphosphoinositides with subsequent production of inositol 1,4,5-triphosphate and diacylglycerol. The former compound releases calcium from the internal stores and the latter is an activator of protein kinase C. Phosphoinositide turnover is followed by the release of histamine from the secretory granules.

Cells prelabeled with  $^{32}\text{P}$ -orthophosphate were stimulated with antigen under conditions which resulted in 38% release of histamine within 10 min and then disrupted at different times. Myosin was recovered selectively by immunoprecipitation with antiplatelet myosin antibodies. Scanning of the heavy chains in coomassie-blue stained polyacrylamide gels revealed 1.1  $\mu\text{g}$  of

myosin per 1,000,000 cells. Quantitative scanning of the autoradiograms obtained from these gels suggested that the amount of radioactive phosphate associated with the myosin heavy chain and light chains increased 2-3 fold over that present in non-stimulated controls during the same time period. Maximal phosphorylation was observed at a time (2.5-10 min) when the calcium signal and rate of inositol phospholipid hydrolysis have been shown to be maximal. Two dimensional maps of tryptic digests indicated that the light chains were phosphorylated by myosin light chain kinase and protein kinase C.

Proposed Course: In order to determine whether phosphorylation is either necessary or sufficient to mediate secretion, we intend to use different agents known to have a specific effect on the activity of MLCK and PKC (Phorbol esters,  $\text{Ca}^{2+}$  etc.) as well as mutant cells lacking key elements known to be involved in mediating secretion.



Molecular Disease Branch  
National Heart, Lung, and Blood Institute  
October 1, 1986 through September 30, 1987

The overall objective of the research program of the Molecular Disease Branch is the elucidation of the molecular and structural properties of the human plasma apolipoproteins (apo), the physiological role of the apolipoproteins and lipoproteins in lipid transport, the determination of the mechanisms involved in the regulation of cellular cholesterol metabolism and transport, and the elucidation of the metabolic and molecular mechanisms involved in plasma lipoprotein synthesis, transport, and catabolism in normal individuals and patients with disorders of lipid metabolism and atherosclerosis.

During the last several years the staff of the Molecular Disease Branch has developed a conceptual framework for the understanding of the dynamic processes involved in the biosynthesis, transport, and catabolism of plasma apolipoproteins and lipoproteins. Within this framework the plasma lipoproteins are conceptualized as a polydisperse collection of lipoproteins, the apolipoprotein composition of which is determined by the laws of mass action. The constituent of the lipoprotein particle which is responsible for the regulation of the lipoprotein particle transport and metabolism is the apolipoprotein moiety. The distribution of a specific apolipoprotein within plasma is governed by the relative concentration of and affinity for the individual plasma lipoproteins. This concept of plasma lipoproteins emphasizes the fundamental importance of the apolipoprotein in regulating metabolism and provides a framework for understanding apolipoprotein-lipoprotein interactions during lipoprotein biosynthesis, transport, and catabolism in normal man and in patients with dyslipoproteinemias and atherosclerosis.

The determination of specific physiological functions of the individual apolipoproteins continues to be of major importance in our understanding of the role of apolipoproteins in lipoprotein structure, function, and metabolism. Based on our current information, apolipoproteins have been shown to be of importance in four general areas of lipoprotein metabolism: 1) cofactor for enzymes (apoC-II for lipoprotein lipase, apoA-I for lecithin cholesterol acyltransferase); 2) ligand on the lipoprotein particle for interaction with high affinity receptor sites (apoB-100 on LDL); 3) exchange protein (apoD) for phospholipids, cholesteryl esters, and triglycerides; and 4) structural component for the lipoprotein particle (apoA-I for HDL, apoB-100 for LDL, and apoB-48 for the chylomicron remnant).

Prerequisite to our understanding of the physiological and biochemical role of apolipoproteins in lipid and lipoprotein metabolism is a detailed knowledge of the molecular structure and function of the plasma apolipoproteins. Over the last several years we have systematically evaluated the molecular structure and primary amino acid sequence of apolipoproteins A-I, A-II, apoC-I, apoC-II, and apoC-III.



During the last two years a great deal of research in the Branch has focused on the B apolipoprotein. ApoB is present in plasma as two isoforms, apoB-100 and apoB-48 of 510,000 and 250,000 daltons in molecular weight respectively. ApoB-100 is the major apolipoprotein in LDL, and interacts with the LDL receptor initiating endocytosis and catabolism of LDL. Initial studies focused on the determination of the complete cDNA and derived amino acid sequence of apoB-100. ApoB-100 is a 4536 amino acid protein containing a cluster of 15 cysteine residues in the amino terminus of the apolipoprotein. ApoB-100 contains 20 potential N-linked glycosylation sites, the majority of which are located in the middle of the apolipoprotein.

A single gene for apoB was localized to chromosome 2 by analysis of human-mouse hybrids. These results established that apoB-100 and apoB-48 were the products of a single gene. Previous studies have proposed that in man apoB-48 and apoB-100 are synthesized by the intestine and liver respectively. Analysis of apoB poly(A)<sup>+</sup> mRNA in the intestine and liver revealed a 14.1 and 7.5 kb mRNA in the intestine and a single 14.1 kb apoB mRNA in the liver. These results provide evidence for the potential synthesis of apoB-100 in the intestine in man. To gain further insight into the apoB isoproteins secreted from the intestine, studies were undertaken utilizing intestinal organ cultures from children ages 1 to 5. Analysis of apoB secreted from these cultures revealed only apoB-100 clearly establishing that apoB-100 may be secreted from intestine. These results are of major importance since apoB-100 containing lipoprotein remnants have been shown to be atherogenic lipoproteins. Previously the liver was considered to be the only site of synthesis of apoB-100 containing lipoproteins. Therefore, lipoprotein remnants from both the intestine and liver may be atherogenic.

The mechanisms for the biosynthesis of two apoB-mRNAs from a single gene is of great interest since the 7.5 kb mRNA terminates in the middle of a long exon in the apoB gene. Therefore differential splicing, a common mechanism for the production of multiple mRNA's from a single gene and nuclear RNA transcript is not operative in the production of the apoB-100 and apoB-48 mRNA. Studies are currently underway to determine the mechanism for the production of the apoB-48 mRNA.

The number and location of the receptor site binding domain(s) on apoB-100 has been of considerable interest. Evaluation of clone and expressed proteins from the apoB-100 gene, and a comprehensive computer analysis of the entire amino acid sequence of apoB-100 have revealed several clusters of positively charged amino acids which are complementary to the consensus sequence of the LDL receptor. A consensus sequence for the LDL binding site on apoB-100 was developed which is complementary to the negatively charged consensus sequence of the LDL receptor binding domains. Several synthetic peptides based on these sequences have been prepared, and recombined with lipoprotein particles to establish if these potential apoB-100 receptor binding





domains are functional. A synthetic peptide of 33 amino acids has been shown to be able to bind to the LDL receptor, and initiate degradation of a lipoprotein particle. Several other synthetic peptides from the amino, middle, and carboxyl terminal regions of apoB-100 are currently under investigation, and results from these studies will provide additional insight into the receptor binding domain(s) of apoB-100 which interacts with the LDL receptor.

To understand the factors which modulate the apoB gene, in vitro studies have been initiated to evaluate potential modulators of apoB gene expression. ApoB gene expression has been studied in normal hepatocytes and HepG2 cells. The levels of apoB mRNA have been quantitated by dot blot hybridization, and apoB concentrations in the media were determined by the Elisa procedure. Initial studies revealed that the apoB mRNA was reduced by incubation of HepG2 cells with LDL, thereby establishing that apoB mRNA levels are regulated by the uptake of LDL through the LDL receptor pathway. Of particular interest, however, was the finding that direct addition of 250H cholesterol to the cells in culture resulted in an increase in apoB in the media, and an increase in apoB mRNA in both normal hepatocytes and HepG2 cells. These results are consistent with the view that cholesterol entering the cells through the LDL receptor pathway or added directly may enter different metabolic pools and have different physiological effects.

The biosynthesis and processing of the human apolipoproteins have been extensively studied. ApoA-I and apoB secreted from HepG2 cells have been shown to be acylated with fatty acids by an ester linkage. Detailed recent studies have shown that plasma apoA-I and apoB are not acylated when quantitated by gas chromatography. Therefore the acylation of apolipoproteins may function primarily in the intracellular processing and transport of the apolipoproteins.

Phosphorylation was also identified as a new post-translation modification of the plasma apolipoproteins. ApoA-I and apoB-100 from both normal hepatocytes and HepG2 cells were shown to be phosphorylated. In vitro apoA-I was able to be phosphorylated by a calcium-calmodulin dependent protein kinase. The phosphorylated amino acid on apoA-I was a serine residue.

The identification of several new post-translational modifications of the plasma apolipoproteins represents the continuation of our systematic analysis of the factors which modulate the biosynthesis, processing and secretion of the plasma apolipoproteins. The identification of these post-translational mechanisms provides new insight into the processes involved in apolipoprotein metabolism. Specific defects in these pathways may ultimately be identified as the site of the molecular defect in patients with dyslipoproteinemias.

The availability of the cDNA sequence and probes for apoB-100 have provide the opportunity to evaluate diseases associated with defects in the B apolipoprotein. Initial studies focused on the molecular defect in abetalipoproteinemia. Abetalipoproteinemia is characterized



The intracellular regulation of cholesterol biosynthesis remains an active area of research within the Branch. The major focus of this research is the study of the short term modulation of the enzymic activity of HMG-CoA reductase by reversible phosphorylation. Both human and rat liver HMG-CoA reductase activity is modulated in vitro and in vivo by a bicyclic cascade system involving two kinases, reductase kinase and reductase kinase kinase. HMG-CoA reductase and reductase kinase undergo reversible activation-inactivation by reversible phosphorylation. The kinase responsible for the reversible phosphorylation of reductase kinase has been designated reductase kinase kinase. Regulation of the enzymic activity of HMG-CoA reductase by a bicyclic cascade system provides a rapid short-term mechanism for the regulation of cholesterol biosynthesis.

Two additional kinases, protein kinase C, and calcium, calmodulin dependent kinase have been shown to phosphorylate HMG-CoA reductase. The calcium, calmodulin dependent kinase has been of particular interest since it is a low molecular kinase (110,000 Da) when compared to the well characterized 660,000 Da kinase. The low molecular weight kinase has a different substrate specificity, subunit protein composition, pattern of autophosphorylation, isoelectric point, and phosphopeptide phosphorylation site suggesting that it is a new unique calcium, calmodulin dependent kinase.

HMG-CoA reductase was shown to be phosphorylated by the low molecular weight form of the calcium, calmodulin dependent kinase with a stoichiometry of approximately two moles of phosphate per mole of the native enzyme.

The regulation of HMG-CoA reductase has now been extended to the investigation of both the short-term and long-term (decreased protein synthesis) of reductase in HepG2 cells by utilizing ligands including LDL and 25 hydroxy cholesterol. These studies will provide the opportunity to directly analyze HMG-CoA reductase mRNA, protein mass, and enzymic activity.

The combined results from this research on the regulation of HMG-CoA reductase activity have clearly established that the regulation is exquisitely controlled and includes mechanisms which modulate the concentration of the enzyme by both transcription and degradation as well as the enzyme activity by reversible phosphorylation utilizing several separate kinase systems.

A central focus of research within the Branch continues to be the analysis of the synthesis, transport, and catabolism of the plasma lipoproteins in normal subjects and patients with dyslipoproteinemias.

ApoE continues to be actively investigated since it plays a pivotal role in lipoprotein metabolism. ApoE is a polymorphic protein in the population with three common isoforms, designated apoE<sub>2</sub>, apoE<sub>3</sub>, and apoE<sub>4</sub>. ApoE<sub>3</sub> is the most common isoform, and is considered to be the normal isoform. Previous studies in our Branch have established that



hydrolyzed and the infused labeled amino acids as well as the native amino acids are quantitated by selective ion monitoring mass spectroscopy. This new method will ultimately provide the ideal method for the quantitation of the synthesis and catabolism of apolipoproteins without the influence of potential artifacts due to iodination, and incomplete sampling of newly secreted lipoproteins.

One of the central focuses of the staff of the Branch is the effective treatment of patients with dyslipoproteinemias particularly those characterized by an increased risk of premature coronary artery disease. Clinical studies focusing on the treatment of patients with hypercholesterolemia have been carried out in the out patient clinic. A systematic analysis of the efficacy of cholestyramine, niacin, neomycin, and mevinolin have been performed. Of particular interest is mevinolin, a competitive inhibitor of HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis. Our recently completed studies with mevinolin on approximately fifty patients has revealed no significant side effects, and there was no evidence of gonadal or adrenal toxicity. Evaluation of long term toxicity and efficacy of mevinolin is currently in process. Mevinolin appears to be a very effective new drug for the treatment of hypercholesterolemia.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02010-16 MDB

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Function of Plasma Lipoproteins and Apolipoproteins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	H. Bryan Brewer, Jr., M.D.	Chief	MDB, NHLBI
OTHERS:	F. Thomas, Ph.D.	Research Chemist	MDB, NHLBI
	A. Hospattankar, Ph.D.	Visiting Associate	MDB, NHLBI
	J. Hoeg, M.D.	Senior Investigator	MDB, NHLBI
	R. Ronan, B.A.	Chemist	MDB, NHLBI
	M. Meng, M.S.	Chemist	MDB, NHLBI
	C. Bishop, B.S.	Chemist	MDB, NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Peptide Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

6.9

## PROFESSIONAL:

3.9

## OTHER:

3.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A consensus sequence of the potential LDL receptor binding domain has been determined based on computer evaluation of the amino acid sequence of apoB-100, and an analysis of monoclonal antibodies which block LDL-LDL receptor interaction. Synthetic peptides based on the sequence of the potential LDL receptor binding domain have been prepared, and have been shown to increase the binding and degradation of apoB-48 lipoproteins incubated with normal human fibroblasts. Additional synthetic peptides are currently being evaluated in order to further define the structure-function requirements for binding of LDL to the LDL receptor.

Human apoB-100 and apoA-I have been shown to be acylated with fatty acid in HepG-2 media. Quantitation of acyl groups on plasma apoB-100 and apoA-I by gas chromatography revealed no fatty acids establishing that circulating plasma apoA-I and apoB-100 are not acylated. Acylation may therefore play an important role in intracellular transport of plasma apolipoproteins.

Analysis of apoB isoproteins secreted from the human intestine revealed that apoB-100 is the primary apoB isoprotein secreted in children ages 1 to 5. Further studies are underway to evaluate the apoB isoproteins secreted in adult intestine.

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Objective:

- 1) Identification of potential binding domains within apoB-100.

Methods

Following the sequence analysis of apoB-100 cDNA, a detailed analysis was undertaken to identify the potential receptor binding domain of apoB-100 for the LDL receptor. Initial studies involved the identification of a fusion protein from a gt-11 expression library which reacted with an apoB-100 monoclonal antibody which blocked the binding of LDL to the LDL receptor. Analysis of the sequence of this fusion protein, and a computer survey of the entire apoB-100 sequence revealed several similar positively charged domains which were complementary to the negatively charged consensus sequence of the LDL receptor. A consensus sequence of the potential LDL binding domain was developed which contained positively charged amino acids at positions 1, 5, and 8, followed by a loop of 8-11 amino acids and two additional positively charged amino acids. The consensus sequence of apoB-100 is as follows: Lys (arg-x-x-x-Lys-x-x-Lys(arg)-loop(8-11 amino acids)-Lys-Lys. Four of these positively charged regions of apoB-100, including one in the amino, middle, and two in the carboxyl terminal portion of apoB-100 have been synthesized by solid phase techniques to gain further information on the interaction of apoB-100 with the LDL receptor. Synthetic peptides of approximately 30 amino acid residues have been reassociated with an apoB-48 containing lipoprotein isolated from a apoE deficient patient, and tested for binding to the LDL receptor on fibroblasts from normal subjects, and patients with familial hypercholesterolemia.

Major Findings:

The binding and degradation of apoB-48 recombined with the synthetic peptides from the carboxyl terminus of apoB-100 containing the potential LDL receptor binding domain were significantly greater than the control apoB-48 lipoproteins. Since the synthetic peptide increased the binding and uptake of apoB-48 lipoproteins it was concluded that the peptide contained a receptor binding domain of apoB-100.

Further studies are underway to evaluate the peptides synthesized from sequences in the amino and middle portions of apoB-100.

Objective:

- 2) Acylation of Plasma ApoA-I and apoB-100

Methods

Previous studies from our laboratory have established that apoA-I and apoB secreted from HepG-2 cells and normal hepatocytes are acylated. These conclusions were based on the finding of radiolabelled palmitate on apoA-I and apoB-100 following NaDodSO<sub>4</sub> gel electrophoresis, 2D gel electrophoresis, and precipitation with monospecific antibodies. The linkage of the fatty



acid to the apolipoprotein was sensitive to treatment with hydroxylamine indicating that the fatty acid was covalently linked to the apolipoprotein by an ester bond.

To determine if the circulating plasma forms of apoB-100 and apoA-I were acylated, both apoA-I and apoB-100 were isolated and the quantity of fatty acid quantitated by gas chromatography. ApoB-100 was isolated by gel permeation chromatography in NaDodSO<sub>4</sub> and apoA-I was isolated by gel and exchange chromatography.

### Major Findings

Extensive analysis of apoB from LDL and apoA-I from HDL by gas liquid chromatography revealed no detectable fatty acids. These results suggested to us that the circulating plasma forms of both apoB-100 and apoA-I do not contain covalently bound fatty acids, thus raising the possibility that fatty acid acylation may be important in intracellular trafficking of the apolipoproteins and/or lipoproteins.

### Objective

3) Analysis of the apoB-100 and apoB-48 isoforms present in the media of organ cultures of the intestines of children ages 1 to 3.

### Methods

Intestinal biopsy samples were obtained from children ages 1 to 3 undergoing study at the Cardiovascular Research Institute in Moscow, USSR. Organ cultures were established, and incubated with radiolabeled amino acids. The secreted apolipoproteins were analyzed by immunoblot and/or NaDodSO<sub>4</sub> gel electrophoresis.

### Major Findings

Only apoB-100 was identified in the media of intestinal organ cultures of infants aged 1 to 3. These results indicate that in the samples assayed only apoB-100 is secreted in the infant, whereas potentially in the adult both apoB-100 and apoB-48 may be secreted. Further studies are in progress to determine the apoB isoforms secreted in organ cultures of adult intestine. It will be critical to definitively establish if both apoB-100 as well as apoB-48 are secreted from the adult intestine since apoB-100 containing lipoproteins are potentially atherogenic.

### Objective:

4) Isolation and characterization of lipoprotein particles containing apoB-48 and apoB-100.

### Methods

Isolated lipoprotein particles containing apoB-48 (LP-B-48) and apoB-100 (LP-B-100) were purified by immunoaffinity chromatography utilizing two monoclonal antibodies (AB-B-3 and AB-B-5) which interact exclusively with



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8. Law, S.W., Lackner, K.J., Fojo, S.S., Hospattankar, A., Monge, J.C., Brewer, H.B., Jr.: The molecular biology of human apoA-I, apoA-II, apoC-II and apoB. In: Angel, A. and Frolich, J. (eds) Lipoprotein Deficiency Syndromes. Plenum Publishing, New York, pp. 152-162, 1986
9. Lackner, K.J., Monge, J.C., Gregg, R.E., Hoeg, J.M., Triche, T.J., Law, S.W., Brewer, H.B., Jr.: Analysis of the apolipoprotein B gene and messenger ribonucleic acid in abetalipoproteinemia. J. Clin. Invest. 78:1707-1712, 1986
10. Schaefer, E.J., Gregg, R.E., Ghiselli, G., Forte, T.M., Ordovas, J.M., Zech, L.A. and Brewer, H.B., Jr.: Familial apolipoprotein E deficiency. J. Clin. Invest. 78:1206-1219, 1986
11. Brewer, H.B., Jr., Anchors, M., Gregg, R.E., Law, S.W.: Recent studies on the molecular defect in apolipoprotein E deficiency. In: Sirtori, C.R., Nichols, A.V., Franceschini (eds) Human Apolipoprotein Mutants. Plenum Publishing, New York, pp. 223-227, 1986
12. Hospattankar, A.V., Law, S.W., Lackner, K., Brewer, H.B., Jr.: Identification of low density lipoprotein receptor binding domains of human apolipoprotein B-100: a proposed consensus LDL receptor binding sequence of ApoB-100. Biochem. Biophys. Res. Comm. 139:1078-1085, 1986



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02012-12 MDB

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Zafarul H. Beg, Ph.D.	Research Chemist	MDB, NHLBI
Others:	J. A. Stonik	Chemist	MDB, NHLBI
	H.B. Brewer, Jr., M.D.	Chief	MDB, NHLBI

## COOPERATING UNITS (if any)

Molecular Disease Branch

## LAB/BRANCH

Peptide Chemistry

## SECTION

NHLBI, NIH, Bethesda, MD 20892

## INSTITUTE AND LOCATION

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

2.0

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have previously established that rat and human hepatic HMG-CoA reductase activity is modulated in vitro and in vivo in a bicyclic cascade system involving reversible phosphorylation of both HMG-CoA reductase and reductase kinase. We have also demonstrated that enzymic activity of HMG-CoA reductase is also modulated in vitro by a protein kinase C-mediated phosphorylation.

Recently, we have purified and characterized a low molecular weight (110,000 Da)  $\text{Ca}^{2+}$ , calmodulin-dependent protein kinase from rat brain cytosol. This kinase phosphorylates histone  $\text{H}_1$ , synapsin 1, and purified HMG-CoA reductase as major substrates. The molecular weight of the holoenzyme, substrate specificity, subunit protein composition, subunit autophosphorylation, subunit isoelectric points, and subunit phosphopeptide analysis suggest that this kinase of M<sub>r</sub> 110,000 may be different from other previously reported  $\text{Ca}^{2+}$ , calmodulin-dependent kinases. Maximal phosphorylation by the low molecular form  $\text{Ca}^{2+}$ , calmodulin dependent kinase of purified HMG-CoA reductase revealed a stoichiometry of approximately one mole of phosphate/mole of 100,000 Da enzyme. Dephosphorylation of phosphorylated and inactivated native and purified HMG-CoA reductase revealed a time-dependent loss of  $^{32}\text{P}$ -bound radioactivity and reactivation of enzyme activity.

Recently in HepG-2 cells we have also investigated the short-term (reversible phosphorylation) and long-term (decreased protein synthesis) control of HMG-CoA reductase by utilizing ligands such as LDL and 25-hydroxycholesterol.

During the past year we have utilized the low molecular weight  $\text{Ca}^{2+}$ , calmodulin-dependent kinase to evaluate the post-translational modification of apolipoprotein A-I by reversible phosphorylation. The reversible covalent phosphorylation of apoA-I may play a pivotal role in apoA-I synthesis and/or metabolism.





Project Description:Objectives:

1) The current research of this laboratory is directed to investigate the short and long-term regulation of HMG-CoA reductase in HepG-2 cells.

Methods Employed:

HepG-2 cells were grown in MEM containing 10% fetal calf serum. After 4-5 days, cells were plated (1:5) on 6-well small dishes (Falcon) and incubated at 37 C for 18 hours in lipoprotein deficient serum (LPDS). At the end of incubation, fresh LPDS (3 ml) with or without ligands (such as LDL, 25-hydroxycholesterol) were added to each well, incubated for different time intervals (15 minutes to 24 hours). At the end of each time interval, cells in each well were washed (x2) with 2 ml of saline containing 50 mM Tris-HCl buffer (pH 7.4). Cells were lysed in a buffer containing 15 mM CHAPS, transferred to a tube and stored frozen at  $-20^{\circ}\text{C}$ . Cells were thawed, vortexed, centrifuged for 1 minute at 12,000 x g. The clear supernatant was utilized for the assay of HMG-CoA reductase activity.

Major Findings:

Incubation of HepG-2 cells for 60-90 minutes with LDL or 25-hydroxycholesterol was associated with inactivation of HMG-CoA reductase activity. This inhibition was reversed by incubation with phosphatase, suggesting the existence of a reversible phosphorylation. Prolonged incubation (beyond 90 minutes) with LDL or 25-hydroxycholesterol revealed a partial to complete irreversible inhibition, suggesting the decline in HMG-CoA reductase protein synthesis and hence in the levels of mRNA. Currently we are quantitating the HMG-CoA reductase mRNA in HepG-2 cells treated with and without LDL and 25-hydroxycholesterol at different time intervals involving both short-and long-term control of HMG-CoA reductase.

Objectives:

2) The current research of this laboratory is also related to the analysis of  $\text{Ca}^{2+}$ , calmodulin-dependent kinase-mediated phosphorylation of purified human plasma apolipoprotein A-I (apoA-I).

Methods Employed:

1) Assay of  $\text{Ca}^{2+}$ , Calmodulin-Dependent Kinase Activity and Human ApoA-I Phosphorylation: Enzyme fractions were assayed in a total volume of 25  $\mu\text{l}$  containing 25 mM MOPS (pH 6.5), 1 mM  $\text{Ca}^{2+}$ , 5 M calmodulin, 6mM  $\text{Mg}^{2+}$ , 0.1 mg/ml purified apoA-I, and 0.05 mM  $[\gamma\text{-}^{32}\text{P}] \text{ATP}$  (specific activity 1700-3400 cpm/pmol). Control incubations lacked  $\text{Ca}^{2+}$  as well as calmodulin and contained 5 mM EGTA. Tubes were incubated at  $30^{\circ}$  for 10 to 60 min or as indicated in each experiment. At the end of the incubation time, duplicate 5  $\mu\text{l}$  aliquots were spotted onto Whatman 3 MM filters discs which had previously been treated with 50  $\mu\text{l}$  of 20% trichloroacetic acid containing 1 mM ATP and 5 mM sodium pyrophosphate, and then three times for 30 min each in 5% trichloroacetic acid. After final wash, discs were dried



in methanol followed by diethylether, and counted in 5 ml of hydroflour (National Diagnostic).

For analysis of [ $\gamma$ - $^{32}$ P]-apoA-I, aliquots were added to tubes containing 1% SDS, 2 mM DTT, 30% glycerol, 0.002% bromophenol blue, and heated in boiling water for 4 min. The proteins were separated by one-dimensional SDS-PAGE, stained with Coomassie blue, dried, and exposed on XAR-5 (Kodak) film at -120 C. Dried gels or autoradiograms were quantitated by densitometric scanning using a Beckman DU-8 spectrophotometer.

Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was performed according to the method of Lemmli with 1.5 mm thick slab gels and the indicated percentage of (acrylamide acrylamide/bisacrylamide ratio of 30/0.8). The molecular weights of the separated subunits were determined by comparison in SDS-PAGE of known molecular weight standards.

### Major Findings:

#### Phosphorylation of Human ApoA-I

Incubation of purified human apoA-1 with  $\text{Ca}^{2+}$ , calmodulin-dependent kinase and ATP-Mg was associated with a time-dependent phosphorylation of apoA-I. Analysis of the  $^{32}$ P-apoA-I by SDS-PAGE revealed a single band containing >95% of the radioactivity in the band corresponding to ApoA-I. Autoradiogram of purified phosphorylated ApoA-I following SDS-PAGE revealed a radioactive band corresponding to the 27,000 Da apoA-I. Phosphorylation of apoA-I in the presence of calmodulin plus EGTA,  $\text{Ca}^{2+}$  alone, or in the absence of  $\text{Ca}^{2+}$  and Calmodulin (plus EGTA), failed to reveal any incorporation of  $^{32}$ P in to apo A-I. These results establish the absence of any other reductase kinases in the purified preparation of  $\text{Ca}^{2+}$ , calmodulin-dependent kinase. Dephosphorylation of  $^{32}$ P-apoA-I with hepatic phosphoprotein phosphatase was associated with a time-dependent loss of radioactivity. No change in the  $^{32}$ P-labeled ApoA-I was observed following incubation with NaF-inactivated phosphatase. Phosphoamino acid analysis of the immunoprecipitated  $^{32}$ P-apoA-I established that phosphorylation occurred at a serine residue.

ApoA-1 secreted from HepG-2 cells as well as primary cultures of human hepatocytes incubated with  $^{32}$ Pi was phosphorylated when analyzed by two dimension gel electrophoresis and by immunoprecipitation with anti-apoA-I monospecific antisera.

### Publications:

1. Beg, Z.H., Reznikov, D.C., and Avigan, J.: Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts by reversible phosphorylation: modulation of enzymic activity by low density lipoprotein, sterols and mevalonolactone. Arch. Biochem. Biophys. 244:310-322, 1986



2. Beg, Z.H., Stonik, J.A., and Brewer, H.B., Jr.: Modulation of the enzymic activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase by multiple kinase systems involving reversible phosphorylation. Metabolism. 36: 1987.
3. Beg, Z.H., Stonik, J.A., and Brewer, H.B., Jr.: Phosphorylation and modulation of the enzymic activity of native and protease cleaved hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase by a calcium, calmodulin-dependent protein kinase. J. Biol. Chem., In press, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02019-09 MDB

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Metabolism of Lipoprotein and Apolipoproteins in Humans

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Richard E. Gregg, M.D.	Senior Investigator	MDB, NHLBI
Others:	Paola Roma, Ph.D.	Visiting Fellow	MDB, NHLBI
	Robert Ross, M.D.	Medical Staff Fellow	MDB, NHLBI
	Jurgen Schaefer, M.D.	Visiting Fellow	MDB, NHLBI
	Diana Hernandez	Chemist	MDB, NHLBI
	Marie Kindt	Chemist	MDB, NHLBI
	Robert Herzog	Biological Aid	MDB, NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB, NHLBI

## COOPERATING UNITS (if any)

Loren A. Zech, M.D.	Senior Investigator	Office of the Dir., NHLBI
Dubo Bojanovski, M.D., Ph.D.	Assistant Professor	Univ. Hanover, FRG

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Peptide Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6.5

## PROFESSIONAL:

4

## OTHER:

2.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

ApoE is a polymorphic protein and two of the isoforms, apoE<sub>3</sub> and E<sub>4</sub>, differ by a single amino acid with apoE<sub>4</sub> having arginine substituted for cysteine. ApoE<sub>4</sub> is catabolized faster than apoE<sub>3</sub>, and this is due to both the addition of a positive charge to apoE<sub>4</sub> and, with the absence of the cysteine, the inability of apoE<sub>4</sub> to form slowly catabolized disulfide dimers. ApoE can bind to LDL receptors and individuals with no LDL receptors have elevated levels of apoE. These elevated apoE levels are due to an increased apoE production rate with a normal catabolic rate. Individuals with apoE<sub>4</sub> have increased LDL levels and this is a result of both increased production and decreased catabolism of LDL.

Subjects with hypoalphalipoproteinemia with an associated restriction fragment length polymorphism of their apoA-I gene have a normal synthetic rate of a structurally and metabolically normal apoA-I with rapid catabolism of apoA-I. Therefore, the causative mutation is likely to be in a gene close to, but not in, the gene for apoA-I.

Abeta and homozygous hypobetalipoproteinemia are characterized by an absence of apoB in plasma. Both types of subjects have a normal apoB gene by Southern blot analysis, and synthesize normal sized apoB mRNA. Abeta subjects have 5 times normal levels of apoB mRNA and increased amounts of apoB protein while hypobeta subjects have 10% of normal levels of apoB mRNA and decreased amounts of apoB protein in hepatocytes. The defect in abeta subjects is likely to be in the post-translational modification and secretion of apoB while the hypobeta subjects are likely to have a structurally abnormal protein that can not be secreted.

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Project Description

The current research of this laboratory is the following:

Objective:

1) To develop the methodology for the quantitation of apolipoproteins by enzyme linked immunosorbent assay techniques.

Methods Employed:

In a competitive solid phase enzyme linked immunosorbent assay the antigen is bound to polystyrene microtiter plate wells followed by the addition of sample or standard and an antibody against the substance to be quantitated. The wells are washed and the quantity of antibody bound to the plates is assayed using an alkaline phosphatase conjugated anti-IgG antibody. From this, the concentration of analyte in the assay sample can be determined.

Major Findings:

ELISA assays for apoA-I, apoA-II, and apoB are currently being performed in the Branch. Because of turnover in personnel, no new assays have been developed this year, but ELISA assays for apoC-II and apoE should be developed during the next year.

Objective:

2) To study the metabolism of apolipoprotein E in normal and dyslipoproteinemic subjects.

Methods Employed:

ApoE kinetics were studied by isolating apoE by ultracentrifugation and column chromatography, chemically modifying the apoE, radioiodinating it with iodine monochloride, reassociating apoE with lipoproteins, followed by injection intravenously into study subjects. Multiple timed plasma samples were obtained, the lipoproteins separated by ultracentrifugation, and the rate of catabolism determined from the radioactive decay curve by computer assisted multiexponential curve fitting.

Major Findings:

ApoE is found in plasma primarily on VLDL and HDL and is important in modulating the catabolism of remnants of triglyceride rich lipoprotein particles. ApoE is a polymorphic protein in the population with three common isoforms, designated as apoE<sub>2</sub>, apoE<sub>3</sub>, and apoE<sub>4</sub>, with apoE<sub>3</sub> being most common in the population and considered to be the normal isoform. An individual can be either heterozygous or homozygous for any of these apoE forms. ApoE<sub>2</sub> is associated with lower than normal levels of LDL cholesterol while apoE<sub>4</sub> is associated with elevated levels of LDL cholesterol. We have previously determined that, compared to apoE<sub>3</sub>, apoE<sub>4</sub> is more rapidly catabolized in humans. In contrast, in vitro receptor binding studies indicate that apoE<sub>3</sub>



and apoE<sub>4</sub> is at amino acid position 112 with apoE<sub>3</sub> having a cysteine at this position and apoE<sub>4</sub> having an arginine. The cysteine is reactive and has been shown to form disulfides with apoE and with other plasma proteins that contain free sulfhydryl moieties. In order to determine if the difference in the catabolism between apoE<sub>3</sub> and apoE<sub>4</sub> was due to the presence of the positively charged arginine in apoE<sub>4</sub> or if it was due to the lack of cysteines and the ability to form disulfides, the following study was performed. ApoE<sub>3</sub> was modified with the addition of either a methyl group or an aminoethyl group to the free sulfhydryl in order to block the sulfhydryl in the first case and to form a positively charged arginine analogue in the second. The in vivo metabolic kinetics of these modified apoE<sub>3</sub> proteins were then compared to apoE<sub>3</sub> and apoE<sub>4</sub>. The aminoethyl apoE<sub>3</sub> was metabolized at the same rate as apoE<sub>4</sub> while the methyl apoE<sub>3</sub> was metabolized at an intermediate rate between apoE<sub>3</sub> and apoE<sub>4</sub>. These results are consistent with the concept that for apoE, both the ability to form disulfides and the electrical charge are important in modulating its metabolism in humans.

LDL cholesterol and apoB levels are elevated in individuals who have apoE<sub>4</sub> in their plasma. In order to determine how apoE<sub>4</sub> kinetically modulates LDL metabolism in humans, LDL apoB kinetic studies were performed in individuals with an apoE<sub>4</sub> phenotype. It was determined that the elevated LDL apoB levels were due to both an increased synthetic rate and a decreased catabolic rate. Therefore, apoE<sub>4</sub> is able to modulate LDL metabolism in humans.

ApoE binds to the LDL receptor in in vitro tissue culture studies and is degraded. In addition, humans with no LDL receptors have 3 to 5 times normal plasma levels of apoE. In order to determine the importance of the LDL receptor in apoE metabolism in vivo, an apoE kinetic study was performed in a subject with LDL receptor negative familial hypercholesterolemia. This subject had a normal apoE catabolic rate with a 3 fold increase in the apoE synthetic rate. Therefore, it is unlikely that the LDL receptor is a major site of apoE catabolism in vivo, and the lack of LDL receptors results in an increased plasma production rate for apoE.

#### Objective:

3) To study the metabolism of normal and variant forms of apolipoprotein A-I in normal and dyslipoproteinemic subjects.

#### Methods Employed:

ApoA-I and the isoforms of apoA-I were isolated from plasma and lymph by ultracentrifugation, column chromatography, and preparative sodium dodecyl sulfate and isoelectrofocusing gel electrophoresis. The isolated isoforms of apoA-I were iodinated by the iodine monochloride method, injected into study subjects, and multiple timed plasma samples obtained. The radiolabeled isoforms of apoA-I were then isolated by ultracentrifugation and preparative isoelectrofocusing. The rate of catabolism of radiolabeled apoA-I isoforms from plasma was determined by computer curve fitting.



Major Findings:

ApoA-I was isolated from a normal subject and from 3 subjects with a restriction fragment length polymorphism (RFLP) linked to apoA-I that co-segregates with hypoalphalipoproteinemia in their families. The metabolism of the isolated normal and hypoalpha apoA-I were then compared in normal subjects. The apoA-I isolated from the two different types of subjects were metabolized at the same rate in the normal subjects. When the kinetics of metabolism of autologous apoA-I were compared in the normal and hypoalphalipoproteinemic subjects, the apoA-I synthetic rates were the same and the decreased apoA-I levels in the hypoalpha subjects were due solely to an increased apoA-I catabolic rate. Since these study subjects synthesize a metabolically normal apoA-I at a normal rate, we conclude that the RFLP of the apoA-I gene associated with hypoalphalipoproteinemia in these families is a marker for an abnormal gene closely linked to, but separate from, the gene for apoA-I.

A family has been identified that has the unusual combination of hyperalpha and hyperbetalipoproteinemia which is vertically transmitted through three generations. These individuals also have xanthomas and premature coronary artery disease. In order to investigate the kinetic etiology of their elevated apolipoprotein levels, the metabolism of apoA-I and LDL apoB were investigated. ApoA-I and LDL were isolated from normal subjects and subjects from this family with combined hyperalpha and hyperbetalipoproteinemia, radioiodinated, and the metabolism of these proteins determined in normal and affected family members. The elevated LDL apoB levels were due to both an increased synthetic rate and decreased catabolic rate, while the increased apoA-I levels were due entirely to increased synthetic rates. Additional genetic and molecular biological studies are being performed to determine the site of the mutation that leads to coordinate regulatory changes in the metabolism of both apoA-I and LDL apoB.

Objective:

4) To investigate the molecular defect in abetalipoproteinemic and hypobetalipoproteinemic subjects that lead to the extremely low to absent circulating plasma apoB concentrations.

Methods Employed:

The presence of apoB on lipoprotein particles is being determined by both immunoblot methods and by a sensitive ELISA assay. Immunohistochemistry methods are being employed to determine the presence of apoB in tissues, while molecular biology methods are being utilized to determine the molecular etiology of the hereditary defect in these subjects.

Major Findings:

In a group of subjects with abetalipoproteinemia and homozygous hypobetalipoproteinemia, Southern blot analysis indicated that there were no major insertions or deletions in the apoB gene. In two different kindreds with abetalipoproteinemia, there was a six fold increase in the level of hepatic apoB mRNA while two unrelated individual with homozygous hypobetalipoproteinemia had 10 to 15% of normal levels of apoB mRNA. Subjects from the two kindreds



with abeta had increased amounts of apoB in their livers by immuno-histochemistry while the two hypobeta subjects had markedly reduced quantities of immunologically detectable hepatic apoB protein. ApoB deficiency states will certainly be associated with multiple etiologies at the gene level, and these results indicate that in at least some abetalipoproteinemic individuals, the primary genetic defect is at the level translational or post-translational modification of the protein that results in an inability to secrete apoB and apoB containing lipoproteins. The molecular defect could be in the primary structure of apoB leading to an altered post-translational modification, or in one of the enzymes necessary for the modification and/or secretion of the newly synthesized nascent apoB protein.

The simplest explanation for the findings in the hypobetalipoproteinemic subjects is a mutation in the coding region of the apoB gene that leads to the formation of an unstable mRNA and an abnormal apoB protein that can not be secreted.

#### Objective:

5) To develop the methodology for performing kinetic investigations of apolipoprotein metabolism with non-radioactive isotopic tracers.

#### Methods Employed:

The non-radioactive isotopic tracer incorporated into amino acids is infused as a bolus injection into normal control and abnormal study subjects. The amino acids are then synthesized into apolipoproteins and the rates of synthesis and catabolism of the proteins are quantitated. Plasma is obtained from the study subjects, the lipoproteins are isolated by ultracentrifugation or column chromatography, the apolipoproteins separated by either high pressure liquid chromatography (HPLC) or gel electrophoresis, the individual proteins hydrolyzed to amino acids, the individual amino acids separated by HPLC, and the ratio of the infused labeled amino acid to the native amino acid determined by selective ion monitoring mass spectroscopy. The kinetics of metabolism of the apolipoproteins are then modeled utilizing the SAAM29 computer simulation program.

#### Major Findings:

This method is in the development stage at the present time. Extensive experience is being obtained and the methods developed for the above type of studies. It has been determined that a bolus infusion of an amino acid is the best way to label the pool of apolipoproteins, and in order to have sufficient isotopic enrichment to be able to detect, a multiply labeled amino acid needs to be used. In addition, we have investigated the stability of the label through the isolation and quantitation procedures. At the present time triple labeled deuterium-leucine and penta labeled <sup>13</sup>C-phenylalanine are the best candidates for the kinetic studies. The apolipoproteins be can isolated in sufficient quantity by HPLC or gel electrophoresis depending on the protein; and negative ionization, selective ion monitoring, mass spectroscopy is a very sensitive method for detecting low levels of isotopic enrichment.





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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02022-07 MDB

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular Lipid and Lipoprotein Biochemistry

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Jeffrey M. Hoeg, M.D.	Senior Investigator	MDB, NHLBI
Others:	H. Bryan Brewer, Jr., M.D.	Chief	MDB, NHLBI
	Juan C. Monge, M.D.	Medical Staff Fellow	MDB, NHLBI
	Stephen Demosky, Jr.	Chemist	MDB, NHLBI
	Santi Datta	Chemist	MDB, NHLBI
	Barbara Winterrowd	Medical Technician	MDB, NHLBI
	Briston Williamson	Lab. Technician	MDB, NHLBI

## COOPERATING UNITS (if any)

Drs. N.N. Tandon, J.T. Harmon, G.A. Jamieson, Red Cross Research Laboratories, Bethesda, MD

Dr. T.E. Starzl, Univ. of Pittsburgh School of Med., Pittsburgh, PA

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Peptide Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

8.2

## PROFESSIONAL:

4.2

## OTHER:

4.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The principal interest of this laboratory is to evaluate cellular lipoprotein and apolipoprotein metabolism. Several different human cells lines are maintained in tissue culture and the ability of cells, lipoproteins, and apolipoproteins from normolipidemic and dyslipidemic patients are studied. Our previous investigations on receptors for low density lipoproteins and high density lipoproteins and the intracellular enzymes relevant to cellular cholesterol homeostasis (acid and neutral cholesteryl ester hydrolase, Acyl: cholesterolacyltransferase, and HMG-CoA reductase) have been extended to evaluate the coordinate regulation of these proteins in human hepatocytes and in the human hepatoma cell line HEPG2. Since the liver is a primary source of apolipoprotein synthesis as well as lipoprotein catabolism, these studies are now focused upon how these different intracellular pathways are related to nascent apolipoprotein biogenesis. Apolipoprotein synthesis is regulated at the levels of transcription and translation. In addition, several of the apolipoproteins are modified post-translationally by glycosylation, fatty acid acylation, co- and post-translational proteolytic cleavage, and by phosphorylation. Insights gained into apolipoprotein synthesis and structure-function relationships are complemented by outpatient clinical trials designed to modify apolipoprotein synthesis and secretion in hyperlipidemic patients.

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Project Description:Objective:

1) To evaluate the interaction of hepatocyte lipid and apolipoprotein synthesis in normal and dyslipidemic patients.

We have previously demonstrated that the human liver expresses receptors for LDL (the B, E receptor), apolipoprotein E, and HDL. These distinct receptors alter hepatocyte cholesterol homeostasis. The possibility of a possible apolipoprotein feedback loop was assessed by studying the effects of incubating both HEPG2 cells (a human hepatoma cell line) and human hepatocytes in primary culture with different media to stimulate and suppress cholesterolgenesis. The associated changes in apolipoprotein secretion into the media as well as changes in the cellular mRNA levels were then studied. In addition, the mRNA levels in the livers of patients with well-defined mutations in lipoprotein metabolism were determined.

Methods Employed:

1) Lipoproteins were isolated by preparative ultracentrifugation and apolipoproteins were isolated by column chromatography.

2) Primary cultures of human hepatocytes and HEPG2 cells were maintained by the techniques we have previously reported.

3) Micro-elisa assays were used to quantitate the low levels of apoA-I and apoB that were secreted into the media of the hepatocytes.

4) Nascent apolipoproteins were also assessed by one and two dimensional gel electrophoresis, immunoprecipitation, and Western blot analysis.

5. Quantitative Dot-blot hybridization and Northern blot analyses were performed utilizing the molecular probes developed within the Molecular Disease Branch.

Major Findings:

1) The expression of apoA-I and apoB mRNA levels can be affected by incubating the cells with various lipoproteins, apolipoproteins, and drugs.

2) The HEPG2 cells generally regulate apolipoprotein gene expression the same as human hepatocytes in primary culture.

3) ApoE mRNA levels in liver from patients with homozygous familial hypercholesterolemia do not always parallel the findings observed with apoB.

4) The apoB mRNA levels in liver from patients with homozygous familial hypercholesterolemia are higher than normal indicating that the hepatocyte apoB regulation is linked to LDL receptor function.





5) Patients with abetalipoproteinemia display sixfold increased apoB mRNA levels despite the absence of apoB in their plasma. The findings suggest a defect in post-translational processing of apoB.

Objective:

2) To determine the characteristic of newly secreted apolipoproteins synthesized by human liver.

Methods:

1) Nascent apolipoproteins were assayed by one and two dimensional gel electrophoresis, autoradiography, immunoprecipitation, and immunoblot analyses.

2) Pulse-chase studies utilizing radiolabeled compounds in defined culture media were conducted.

Major Findings:

1) Human liver produces only apoB-100 mRNA and protein.

2) In contrast, in children human intestine directly secretes only apoB-100 despite the fact that it produces mRNA for both apoB-100 and apoB-48.

3) Both apoA-I and apoB undergo fatty acid acylation as assessed in the media of HEPG2 cells and human hepatocytes. However, the circulating forms of these proteins in the plasma of normolipidemic individuals contain no fatty acids.

4) Both apoA-I and apoB are phosphorylated by HEPG2 cells and by human hepatocytes.

Objective:

3) The treatment of patients with inborn errors of apolipoprotein metabolism.

1) Randomized, placebo-controlled clinical trials were continued using Lovastatin (Mevinolin) in the treatment of type II hyperlipoproteinemia.

2) Hormone studies evaluating the hypothalamic-pituitary-testicular axis in men were completed.

3) The followup evaluations of the plasma lipoproteins and the coronary artery disease in homozygous familial hypercholesterolemia post-liver transplantation were conducted.

4) Of the six prospective, double-blind, randomized, placebo-controlled trials we have conducted over the past 7 years, the most effective drugs in the treatment of type II hyperlipoproteinemia are the bile acid sequestrant



cholestyramine and lovastatin. Lovastatin (mevinolin) was the most easily tolerated preparation with the fewest side effects.

5) Liver transplantation virtually normalizes the plasma lipoprotein concentrations in homozygous familial hypercholesterolemia. In addition, it appears that it stabilizes and perhaps induces regression of coronary atherosclerosis within 18 months.

#### Publications:

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13. Hoeg, J.M., Maher, M.B., Bailey, K.R., Brewer, H.B., Jr.: Comparison of 6 pharmacological regimens for hypercholesterolemia. Am. J. Cardiol. 59:812-815, 1987
14. Brewer, H.B., Jr., Law, S.W., Gregg, R.E., Hoeg, J.M.: The Genetic Human Dyslipoproteinemias. Recent Advances in Arteriosclerosis Research. Hauss, W. H., Wissten, R.W., Gruenwald, J., Eds., Westdeutscher Verlag pp 17-27, 1987
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18. Laue, L., Hoeg, J.M., Barnes, K., Koss, J.L., Loriaux, L., Chrousos, G.P.: Abnormal growth hormone secretory dynamics in children with familial hypercholesterolemia: Hormone Research (accepted for publication).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02024-06 MDB

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular biology of plasma apolipoproteins and lipoproteins

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Simon W. Law, Ph.D.	Senior Investigator	MDB, NHLBI
Others:	H. Bryan Brewer, Jr., M.D.	Chief	MDB, NHLBI
	Silvia Fojo, M.D., Ph.D.	Medical Staff Fellow	MDB, NHLBI
	Stephen Grant, M.D.	Medical Staff Fellow	MDB, NHLBI
	Keiichi Higuchi, Ph.D.	Visiting Fellow	MDB, NHLBI
	Ashok Hospattankar, Ph.D.	Visiting Scientist	MDB, NHLBI
	Juan Monge, M.D.	Medical Staff Fellow	MDB, NHLBI

## COOPERATING UNITS (if any)

A. Sakaguchi & S. Naylor - Departments of Medicine and Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, Texas

## LAB/BRANCH

Molecular Disease Branch

## SECTION

Peptide Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

7.3

## PROFESSIONAL:

6.3

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have determined the entire mRNA sequence of human liver apolipoprotein (apo) B-100, the ligand on low density lipoproteins (LDL) which interacts with the LDL receptor and initiates receptor mediated endocytosis and LDL catabolism. At 4536 amino acids, apoB-100 is the largest protein cloned and sequenced. The availability of cDNA clones have enabled us to:

1. Predict a low amphipathic helical structure of apoB-100, an unusual feature when compared to other human apolipoproteins.
2. Propose multiple LDL receptor binding domains in apoB-100.
3. Localize the apoB-100 gene to the p23 pter region of human chromosome 2.
4. Determined there is one gene copy per haploid genome.
5. Analyze genetic defects in apoB structure in patients with dyslipoproteinemias such as familial abetalipoproteinemia and hypobetalipoproteinemia.
6. Study the expression of the apoB-100 gene in-vitro.
7. To determine structural relationship between apoB-100 and apoB-48.

We have also initiated studies on the regulation of human apoA-I gene. We have determined the region between the cap site and 560 bps 5' upstream contain all the information required for tissue specific expression of apoA-I by way of CAT assay of transient expression of transfected apoA-I promoter constructs. The methodologies will be applied to normal as well as abnormal apolipoprotein genes.

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Project Description:Objectives:

- 1) Identification of low density lipoprotein receptor binding domains of human apoB-100.
- 2) Chromosome assignment and copy number of apoB-100 gene.
- 3) Analysis of the apoB-100 gene in patients with abetalipoproteinemia.
- 4) Evaluate the apoB gene expression in human intestine.
- 5) Analysis of the human apoA-I promoter.

Methods Employed:

- a) Purification of fusion proteins: gt-11 recombinant clones were lysogenized and induced with 0.2mM IPTG to synthesize the fusion protein. Cell extract was fractionated by preparative SDS-PAGE and the fusion protein electroeluted.
- b) Immunoblot Analysis: Fusion protein was analyzed by SDS-PAGE in 7.5% acrylamide gel and electrophoretically transferred to nitrocellulose paper. Monoclonal antibodies ABB-1, ABB-3, and ABB-5 were purchased from Radioimmunoassay Inc. Standard procedures for immunoreactions were followed.
- c) ApoB-100 cDNA probes: ApoB-100 cDNA clones were isolated from human liver cDNA libraries established in plasmid or in lambda phage. cDNAs were radiolabelled by nick-translation.
- d) Human-Mouse somatic cell hybrids: The human-mouse somatic cell hybrids were constructed by fusing mouse LM/TK or RAG fibroblasts with human fibroblasts or leukocytes.
- e) Filter hybridization: All RNA and DNA filters were prepared and hybridized to apoB-100 cDNA probes by established procedures.

Major Findings:

- 1) A fusion protein induced from an apoB gt-11 clone reacted with apoB-100 monoclonal antibodies known to block the binding of LDL to the LDL receptor. The fusion protein contains an amino acid sequence domain enriched in positively charged residues which is complementary to the negatively charged amino acids present in the consensus LDL receptor binding domain. This sequence of apoB-100 is proposed as a binding domain for the interaction with the LDL receptor. Comparison of derived amino acid sequences from the entire structure of the apoB-100 molecule revealed several similar domains enriched in positively charged amino acids. A consensus sequence of the potential LDL binding domain was identified



which contained positively charged amino acids at positions 1, 5 and 8 and a loop of 8-11 amino acids followed by two adjacent positively charged amino acids. These results are interpreted as indicating that there are several potential LDL receptor binding domains in apoB-100.

2) The gene for apoB-100 has been localized to the p23 pter region of chromosome 2 by filter hybridization analysis with radiolabelled apoB-100 cDNA probes and human-mouse somatic cell hybrids containing chromosome 2 translocations. The localization of the apoB-100 gene completes the genomic organizational relationship of the LDL receptor and the two apolipoprotein ligands for the LDL receptor, apoE and apoB-100; the LDL receptor and apoE having been previously localized to chromosome 19. We have also determined that there is only one apoB gene copy per haploid genome.

3) The apoB-100 gene in leukocytes and the apoB-100 mRNA and translated apolipoprotein in the livers from normal and abetalipoproteinemic individuals were evaluated. Four cDNA probes for apoB-100 covering the 5', middle, and 3' regions of the apoB-100 mRNA were utilized and Southern blot analysis indicated that the apoB-100 gene is present in abetalipoproteinemia without major insertions or deletions. Polyadenylated hepatic apoB-100 mRNA from two abetalipoproteinemic patients was normal in size, and the concentration of apoB-100 mRNA was increased six fold compared with control hepatic apoB-100 mRNA levels. ApoB-100 was detected in hepatocytes of abetalipoproteinemic patients by immunohistochemical techniques. These results indicate that the biochemical defect in abetalipoproteinemic patients studied is most consistent with a post-translational defect in apoB-100 processing or secretion with an up-regulation of the apoB-100 mRNA.

4) Northern blot analysis of human liver and intestine mRNA revealed two separate apoB mRNA of 14.1 and 7.5 kb in the intestine, and a single 14.1 kb apoB mRNA in the liver. cDNA probes which encode for the 5', middle and 3' regions of the human apoB-100 mRNA have been utilized to evaluate the number of apoB genes present in the human genome by Southern blot hybridization analysis. Comparison of restriction enzyme digestions of high molecular weight leukocyte DNA and an apoB genomic clone with cDNA probes were consistent with only a single apoB gene per human haploid genome. Further analysis with synthetic oligonucleotides definitively established that there is a single apoB-100 gene. Further blotting analysis showed sequence homology to apoB-100 mRNA up to the region encoding amino acids 2000-2100. The two mRNA species observed in the intestine must be derived from a single nuclear apoB RNA transcript.

5) ApoA-I is synthesized in the liver and intestine in man. We have analyzed the tissue-specific expression of the apoA-I gene. A 2.5 kb 5' flanking region of the human apoA-I gene has been isolated and characterized. The transcription start site was determined by primer extension and is 235 bp upstream from the AUG start codon. A 560 bp fragment of the 2.5 kb flanking region from -490 to 71 was fused upstream to the chloramphenicol acetyltransferase (CAT) gene and introduced into human oral epidemoid carcinoma cells (KB) mouse 3T3 cells, chinese hamster



ovary cells (CHO), human hepatoma cells (HepG2) and human duodenum carcinoma cell (Hutu80). After DNA transfection, CAT activity was observed only in HepG2 and Hutu80. The level of CAT activity of the -49 to 71 construct was the same as the 2.5 kb 5' fragment of apoA-I. Thus, the 560 bp fragment contains the control elements which moderate tissue-specific human apoA-I expression.

#### Publications:

1. Law, S.W., Grant, S.M., Higuchi, K., Hospattankar, A., Lackner, K., Lee, N. and Brewer, H.B. Jr.: Human liver apolipoprotein B-100 cDNA: Complete nucleic acid and derived amino acid sequence. Proc. Natl. Acad. Sci. 83: 8142-8146, 1986.
2. Hospattankar, A.V., Law, S.W., Lackner, K. and Brewer, H.B. Jr.: Identification of low density lipoprotein receptor binding domains of human apolipoprotein B-100: A proposed consensus LDL receptor binding sequence of apoB-100. Biochem. Biophys. Res. Commun. 139: 1078-1085, 1986.
3. Higuchi, K., Monge, J.C., Lee, N., Law, S.W., Brewer, H.B. Jr., Sakaguchi, A.Y. and Naylor, S.L.: The human apoB-100 gene: ApoB-100 is encoded by a single copy gene in the human genome. Biochem. Biophys. Res. Commun. In press.
4. Lackner, K., Monge, J.C., Gregg, R.E., Hoeg, J.M., Triche, T.J., Law, S.W. and Brewer, H.B. Jr.: Analysis of the Apolipoprotein B gene and messenger ribonucleic acid in abetalipoproteinemia. J. of Clin. Invest. 78: 1707-1712, 1986.
5. Fojo, S.S., Law, S.W., and Brewer, H.B. Jr.: The human preproapolipoprotein C-II gene: Complete nucleic acid sequence and genomic organization. FEBS Letters. 213: 221-226, 1987.
6. Law, S.W., Lackner, K., Fojo, S.S., Hospattankar, A., Monge, J.C., and Brewer, H.B. Jr.: The molecular biology of human apoA-I, apoA-II and apoB. Lipoprotein Deficiency Syndromes. A. Angel and J. Frohlich (Eds). Plenum Publishing Corp. 1986, pp 151-162.
7. Brewer, H.B. Jr., Bojanovski, D., Gregg, R.E., and Law, S.W.: Recent studies on the metabolic defect in Tangier disease in: Human Apolipoprotein Mutants. C.R. Sitori, A.V. Nichols and G. Franceschini (Eds). Plenum Publishing Corp. 1986, pp 129-132.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02028-03 MDB

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Biology of the ApoC-II Gene

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Silvia S. Fojo, Ph.D., M.D.	Medical Staff Fellow	MDB, NHLBI
Others:	Simon W. Law, Ph.D.	Senior Staff Fellow	MDB, NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB, NHLBI

## COOPERATING UNITS (if any)

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## LAB/BRANCH

Molecular Disease Branch

## SECTION

Peptide Chemistry

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

2.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- |   |  |                                      |
|---|--|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |                                      |
| <input type="checkbox"/> (a2) Interviews    |  |                                      |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The complete genomic sequence of normal human apoC-II has been elucidated from an apoC-II clone isolated from a human placental genomic DNA phage library. The normal apoC-II gene consists of 3407 base pairs and like the genomic structure of other known apolipoprotein genes it contains 3 introns and 4 exons.

DNA isolated from the WBC of 6 different patients with apoC-II deficiency was isolated and analyzed by Southern blotting using an apoC-II cDNA probe. The restriction endonuclease digestion pattern in 5 patients were normal but Southern blot analysis of one patient showed a major rearrangement in one of the apoC-II alleles suggesting there is a large insertion or deletion present.

The apoC-II gene from two of our patients with apoC-II deficiency has been cloned into an EMBL-3 lambda vector genomic library. Several apoC-II clones have been isolated from each library and determination of the complete genomic structure of the potentially defective apoC-II gene is underway to determine the specific molecular defect.

Total RNA from the liver of one of the patients with apoC-II deficiency has been isolated. Northern blot analysis reveals a normal size apoC-II mRNA and quantitation by slot blot analysis shows near normal levels of apoC-II mRNA in this patient suggesting the possibility of a post-translational defect.

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Project Description:Objective:

- 1) Structural analysis of the apoC-II gene.

Methods Employed:

A human placental genomic DNA phage library made by infecting E. Coli 600 with charon 28 recombinant phage was obtained from Dr. P. Leder. The library was screened for apoC-II clones by using an apoC-II cDNA, 350 bp-Alu I restriction fragment as probe. A 5 kb BamHI and 2.7 kb EcoRI fragment of insert genomic DNA isolated from phage containing the apoC-II insert were subcloned into pBR322 and then sequenced by the chemical cleavage method of Maxam and Gilbert and/or the dideoxynucleotide chain termination method of Sanger.

Major Findings:

The complete nucleic acid genomic sequence of normal human preproapoC-II has been determined. The apoC-II gene consists of 3407 bases and contains 4 exons and 3 introns. Exon 1 consists of 25 nucleotides and encodes most of the 5' untranslated region of the apoC-II mRNA. Exon 2 is 68 bp-long and encodes the rest of the 5' untranslated region of the apoC-II mRNA and amino acids - 28 through - 11 of the signal peptide. The third (160 bp) and fourth (231 bp) exons encode amino acids -10 to +44 and +45 to the polyadenylation site of the apoC-II gene, respectively. This organization is very similar to and supports the concept that at least some of the apolipoprotein genes evolved from a common ancestor. Unusual features of the apoC-II gene include a long first intron of 2495 bases which contains 4 Alu type repetitive sequences and a 22 dinucleotide sequence of GT repeats. The third intron also contains a 38 bp-long sequence that is repeated 6 times within this intron.

Objective:

- 2) Analysis of the apoC-II gene in patients with apoC-II deficiency.

Methods Employed:

Genomic DNA from 6 patients with apoC-II deficiency was isolated from WBC and digested with restriction endonuclease BamHI, BglI, EcoRI, Hind III, PstI, SphI, SstI. The restriction fragments were run on 8.7% slab agarose gel electrophoresis and transferred to nitrocellulose paper for Southern blotting. Hybridization was performed by utilizing a 350 bp apoC-II cDNA probe.

WBC genomic DNA from 2 patients with apoC-II deficiency was partially digested with MboII and used to prepare a genomic library by infecting coli P2392 cells with EMBL-3 recombinant phage. The library was screened for apoC-II cDNA clones by using an apoC-II cDNA probe. DNA was prepared from the isolated clones and sequenced by the dideoxynucleotide chain termination method of Sanger.



Total RNA from normal and one apoC-II deficient patient's liver was isolated by the guanidinium thiocyanate method. Hybridization following Northern and slot blot analysis was performed by utilizing a nick-translated probe isolated from an apoC-II cDNA and an albumin cDNA clone as control. Densitometric scanning of the autoradiogram was then performed.

#### Major Findings:

Southern blot analysis of the DNA of 5 patients with apoC-II deficiency shows normal restriction enzyme pattern of the apoC-II gene digested with BamHI, BglI, EcoRI, HindIII, PstI, SphI, and SstI resulting in a 5 Kb, 6Kb, 3.6Kb, 23 Kb, 3.4Kb, 6Kb, 0.8 Kb fragments respectively. Analysis of genomic DNA of 1 patient, however, shows both normal and abnormal sized fragments (in 5 out of 7 restriction enzymes) suggesting this patient is a compound heterozygote with one apoC-II allele having a normal size and the other allele having undergone a major rearrangement.

We have cloned the apoC-II gene of this patient as well as that of another patient with normal restriction enzyme pattern by Southern blotting. We have isolated several clones for each library and have started sequencing in order to define the molecular defect that results in lack of expression of the apoC-II gene in these patients.

Northern blot analysis of total liver RNA from one patient with apoC-II deficiency shows a normal size apoC-II mRNA of 500 bp. Quantitation by slot blot analysis reveals near normal levels of the apoC-II message. Thus, there is preliminary evidence that this patient may have a defect at the post translational level.

#### Reference

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2. Law, S.W., Lackner, J.J., Fojo, S.S., Hospattankar, A., Monge, J.C., Brewer, H.B., Jr.: The molecular biology of human apoA-I, apoA-II, apoC-II and apoB. In: Angel A. and Frolich, J. (eds) Lipoprotein Deficiency Syndromes. Plenum Publishing, New York, 1986 p 151-162.
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Annual Report of the  
Laboratory of Molecular Hematology  
National Heart, Lung, and Blood Institute  
October 1, 1986 to September 30, 1987

The Laboratory of Molecular Hematology (LMH) is composed of three sections: the Section on Molecular Genetics is primarily involved in developing the basic knowledge and technology for carrying out gene therapy for human genetic diseases; the Section on Molecular Cloning is primarily concerned with understanding the nature of transcriptional control elements; and the Section on RNA and Protein Biosynthesis is primarily concerned with understanding the mechanism and regulation of eukaryotic gene expression at both the transcriptional and translational levels.

SECTION ON MOLECULAR GENETICS

The disease chosen as the initial candidate for human gene therapy is adenosine deaminase (ADA) deficiency, a cause of severe combined immunodeficiency (SCID). Retroviral techniques and recombinant DNA technology have been used to construct retroviral vectors containing the human ADA gene as well as a selectable gene, NeoR (the latter codes for a phosphotransferase enzyme that confers resistance to the drug G418, a neomycin analogue that can kill mammalian cells). A highly efficient procedure for transferring functional genes into mammalian tissue culture cells in vitro and into bone marrow cells of mice in vivo was developed last year using these retroviral vectors as a delivery system.

When murine hematopoietic progenitor cells are infected in vitro with a vector carrying the NeoR gene and then reinjected into a lethally irradiated recipient mouse, 85-90% of the stem cells (CFU-S) can be shown to carry an intact copy of the NeoR gene. The majority of these cells can be shown (by analyzing spleen foci in the CFU-S assay) to produce the NeoR phosphotransferase (NPT). Using the knowledge gained from the murine system, an autologous bone marrow transplantation (BMT)/gene transfer protocol has been developed this year for nonhuman primates. These latter studies have been done in collaboration with the Clinical Hematology Branch, NHLBI, for studies with the rhesus monkey, and in collaboration with the Bone Marrow Transplantation Program at the Memorial Sloan-Kettering Hospital, New York City, for studies with Cynomolgus macaque. Expression of the human ADA and the prokaryotic NeoR genes at low levels has been demonstrated in several monkeys.

During the past year, this Section has achieved the following results:

(1) The human ADA gene (as well as the NeoR NPT gene) has been efficiently expressed in approximately 0.5% of the circulating mononuclear cells of one monkey and at lower levels in several other animals. The autologous BMT/gene transfer protocol that is being developed with the retroviral vector SAX is projected for use, once it is sufficiently tested, in human gene therapy clinical trials for ADA deficiency. Greater efficiency and reproducibility are still required.

(2) In utero gene transfer and expression have been demonstrated in the fetal lamb. In a collaborative study with Dr. Esmail Zanjani, Minneapolis, and Drs. Michael Harrison and Alan Flake, San Francisco, a sheep in utero



transplantation/gene transfer protocol has been successfully developed. Peripheral blood was removed from a 96 day old fetal lamb, infected in vitro with a retroviral vector, N2, carrying the NeoR NPT gene, and reinfused back into the donor fetus. After the lamb was born, bone marrow studies indicated that the NeoR gene was present and functioning.

(3) Human hematopoietic progenitor cells can be infected with the vector SAX and are resistant to G418 in a CFU-C assay at an efficiency of 1-2%. In like manner, bone marrow cells from patients with ADA deficiency can also be infected with the SAX vector. These genetically defective cells also were shown to express the NeoR gene of the SAX vector in 1-2% of the CFU-C progenitor.

## SECTION ON MOLECULAR CLONING

To understand the nature and position of key transcriptional control elements which regulate differential control of gene expression, specific synthetic DNA control sequences are being constructed and their affects on transcription examined. Tissue-specific promoter elements are also being used to increase gene expression in retroviral vectors used to mediate specific gene transfer.

Proteins purified by affinity chromatography using specific synthetic DNA sequences are being used to identify transcription factor interactions with DNA control sequences and their effects on topology which regulate transcriptional initiation.

During the past year this section has:

(1) Developed rapid and efficient procedures for the synthesis and purification of specific DNA promoter elements.

(2) Constructed multicopy tandem head-to-tail arrays of specific transcriptional control sequences which have been used to a) extensively purify polypeptides required for accurate transcription, b) generate probes to identify clone and sequence translation factor genes, c) alter the structure of retroviral envelope glycoproteins to direct the tissue specific targeting of this gene vector system.

(3) Characterized the length and sequence requirements of donor DNA required for optimal T4 DNA ligase activity.

(4) Developed procedures for coupling both linear and supercoiled plasmids containing multicopy Ad2 major late promoter inserts, to a cellulose matrix for large scale purification of DNA-binding proteins.

## SECTION ON RNA AND PROTEIN BIOSYNTHESIS

To understand the regulation of gene expression by RNA polymerase II, plasmids containing multiple repeats of promoter elements of the Adenovirus 2 major late transcription unit are being used to fractionate active HeLa, K562, and liver nuclear extracts into individual factors required for correct initiation.





The DNA binding properties of individual factor binding to specific promoter elements, and their interaction(s) are being investigated.

The mechanisms by which adenoviruses and influenza viruses take over the translational machinery of the infected cell, and the ability of certain cell lines to prevent viral takeover, are being studied.

The role of eIF-2B during protein synthesis initiation in normal and viral infected cells is being studied using monoclonal as well as polyclonal antibodies directly against eIF-2B and factors whose activities it modulates.

During the past year this section has:

- (1) Identified and extensively purified a transcription initiation factors which specifically binds to the upstream promoter sequence (UPS) of the adenovirus 2 major late promoter (Ad2 MLP).
- (2) Developed a radiolabel transfer procedure based on UV crosslinking which can directly identify sequence-specific DNA binding proteins.
- (3) Identified a new transcription factor and its cognate promoter element which regulates the efficiency of Ad-2 MLP transcription.
- (4) Identified isoforms of the UPS binding factor which have distinct DNA binding and transcriptional activities.
- (5) Developed a novel assay system for monitoring the transcriptional activity of individual trans-acting factors required for correct initiation by RNA polymerase II.
- (6) Demonstrated that monoclonal antibodies prepared against the 82 kDa subunit of eIF-2B will directly inhibit initiation of translation by interference with its guanine nucleotide exchange activity for eIF-2.
- (7) Correlated binding of VAI RNA to the interferon-induced P68 protein kinase with prevention of its activation by double-stranded RNA.
- (8) Identified and isolated 3 independent genomic clones for the alpha subunit of eIF-2. Sequencing and identification of transcription promoter elements are in progress.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02213 10 MH

## PERIOD COVERED

October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Identification and Regulation of Factors Required for Transcription by RNA Polymerase II

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Safer, Medical Officer, LMH, NHLBI W. Anderson, Chief, LMH, NHLBI

Others: J.A. Thompson, Expert, LMH, NHLBI

S. Sturm, Staff Fellow, LMH, NHLBI

R. Cohen, Staff Fellow, LMH, NHLBI

S. Garfinkel, Bio. Lab. Tech., LMH, NHLBI

T. Boal, Biol. Lab. Tech., LMH, NHLBI

L. Yang, Biologist, LMH, NHLBI

K. Anderson, Guest Worker, LMH, NHLBI

COOPERATING UNITS (if any) Michael Katze, Memorial Sloan-Kettering Cancer Center, N.Y., NY  
Tom Shenk, Princeton University, Princeton, NJ; Rosemary Jagus, University of  
Pittsburg, Pittsburg, PA

## LAB/BRANCH

Laboratory of Molecular Hematology

## SECTION

Section on RNA and Protein Biosynthesis

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

9.6

## PROFESSIONAL:

4.6

## OTHER:

5.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Regulation of gene expression occurs at the level of transcription, processing, transport, and mRNA translation. The primary goal of this section is to investigate the transcriptional and translational control mechanisms responsible for regulated gene expression.

To identify components required for transcription of genes by RNA polymerase II, transcriptionally active nuclear extracts were fractionated using FPLC and DNA affinity chromatography. Sequence-specific DNA binding factors required for transcription of the adenovirus 2 major late promoter were identified by DNase I footprinting, mobility shift assays, and their function(s) studied using a novel assay system based on binding to synthetic oligonucleotides of their cognate promoter sequences. In addition, a UV protein-DNA crosslinking procedure was developed which is able to directly identify sequence specific DNA-binding factors at very early stages of their purification. Using these methods, trans-acting factors binding to new Ad2 MLP promoter elements in addition to those recognizing the TATAA and upstream promoter sequence (UPS) have been identified.

During infection by adenoviruses and influenza viruses, activation of host cell ds-RNA dependent eIF-2  $\alpha$  kinase is prevented by VAI RNA and an unknown, but functionally similar influenza gene product. The mechanisms by which certain cell lines escape viral takeover of their translational factors which participate in virus-host interactions, the genes for subunits of eIF-2 and eIF-2B are being identified and sequenced. In addition, binding of VAI RNA to interferon-induced P68 eIF-2 $\alpha$  kinase has been shown to inhibit activation by double-stranded RNA.

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Project Description:

Objectives: The major goals are (1) to determine the sites and mechanisms of transcriptional and translational control of gene expression; (2) to identify and characterize the components involved in such regulation; (3) to develop active in vitro translation and transcription systems which retain key regulatory features found in intact cells.

Methods Employed:

Conventional ion exchange and gel permeation chromatography, as well as FPLC and HPLC adaptations, are used to rapidly isolate transcription factors as individual components or as complexes with multicopy promoter elements and/or RNA polymerase II. Multicopy promoter element constructs are being used to affinity purify their cognate DNA binding proteins. Transcription factors are identified as protein factors required for de novo assembly of active transcription complexes on the Adenovirus 2 major late promoter or as proteins which restore transcription to cell extracts specifically depleted of individual components by DNA affinity chromatography. Specific binding of transcription factors to promoter elements is being determined by mobility shift, footprinting and exonuclease digestion procedures. A modified UV DNA-protein cross-linking procedure has been developed to directly identify sequence specific promoter binding factors. In vitro immunization procedures for monoclonal antibody production are being applied for the eventual use in factor purification and in function studies.

Probes based on N-terminal amino acid sequence information of eIF-2 are being used to screen genomic and cDNA libraries. The selected clones are evaluated via restriction enzyme mapping, hybridization-selected mRNA translation and partial sequence analysis. Once the desired genes have been isolated, sequence analysis and electron microscopic R-loop analysis can be utilized to elucidate the genetic organizational structure and the sequences responsible for the primary structure of the eIF-2 protein and the transcriptional regulation of the gene. mRNA is measured by hybridization to specific probes generated using Sp6 and M13 vectors. Hybrids are analyzed by dot blot and nuclease protection mapping. Functional mRNA translational intermediates are fractionated by sucrose density gradient centrifugation.

Major Findings:

1. Three proteins which bind to the adenovirus 2 major late upstream promoter sequence (Ad2 MLP UPS) have been identified and extensively purified. A 116 kDa polypeptide appears to be the precursor for a more abundant 45 kDa protein. The latter exists as two distinct isoforms, one of which has high affinity for the UPS, but is transcriptionally inactive, while the second is transcriptionally active, but has lower DNA binding activity. The mechanisms by which these forms may be interconverted is currently being studied.
2. A new UV cross-linking protocol has been developed with high specificity for DNA binding proteins which bind to specific promoter elements. This has allowed the identification of sequence-specific DNA binding proteins which can not be demonstrated by footprinting or mobility shift techniques. The transfer



of  $^{32}\text{P}$  from phosphodiester bonds within the binding site to the cognate factor also allows relationships between DNA binding proteins to be explored.

3. A novel affinity chromatographic procedure was developed for the Ad2 MLP binding factor. Multicopy UPS containing DNA fragments incubated with crude preparations of the factor alters the chromatography of the factor on Mono Q resin. Gradient salt elution then is used to elute the specific UPS binding factor at a much higher salt than it would normally elute. This modified DNA affinity technique can be successfully utilized in relatively crude fractions which still contain nucleases which preclude conventional solid support DNA affinity columns.

4. Depletion of single sequence-specific DNA binding proteins from transcriptionally active K562 or HeLa nuclear extracts can be achieved by precipitation with DNA fragments containing multicopy cognate promoter elements. Alternatively, functional sequestration of unique factors can be achieved by addition of single oligonucleotides containing promoter binding sequences. This readily allows a functional assay to be established for any transcriptionally active DNA binding protein.

5. Two new promoter elements which regulate in vitro transcriptional activity of the Ad2 MLP have been identified. One binds topoisomerase I and is located at the 5' border of the UPS. A functional significance for this topo I binding site is indicated by a high extent of occupancy determined by UV cross-linking and mapping of topo I cleavage sites within Ad2 MLP. A second promoter element has been mapped downstream of the cap site. Mutational analysis of effects on DNA binding and transcriptional activities suggest a positive role of each element.

6. Three genomic clones for eIF-2 have been identified and isolated. These are currently being sequenced and analysed for promoter elements which regulate their expression.

7. The interaction of VAI RNA with the interferon-induced double-stranded RNA activated protein kinase (P68) has been studied. Increased activity of the kinase in cells infected with adenovirus mutants which do not produce VAI RNA is the result of kinase activation, not increased synthesis.

8. Purified VAI RNA can block this activation of P68. This may be mediated by the formation of VAI RNA-p68 protein complex, which can be demonstrated both in vitro and in vivo.

#### Proposed Course of the Project:

1. Polypeptides identified as components of RNA polymerase II initiation and elongation complexes will be purified from transcriptionally active nuclear extracts of HeLa, liver, and K562 cells. Monoclonal antibodies will be prepared against transcription complex components and will be tested for inhibition of transcriptional activity. The specific targets of these antibodies will then be purified by a large scale affinity chromatography. The ultimate goal will be to assign specific functions to these transcriptional components required for the initiation process.





2. Binding sites for specific and general transcription factors will be defined and compared for the Adenovirus 2 major late promoter, human and mouse and globin genes, and the eIF-2d gene. Similarities and differences among the regulatory regions of these genes will increase our understanding of how these genes are differentially regulated.

3. Once DNA binding sites for specific factors have been defined, purification by DNA affinity chromatography will be used to purify factors sufficient to perform both physical and functional studies.

4. Covalent modifications of transcription factor which regulate their activity will be examined.

#### Publications:

1. Curcio, M.J., Kantoff, P., Schafer, M., Anderson, W.F., and Safer, B.: Compensatory increase in levels of <sup>minor</sup> globin in murine  $\beta$ -thalassemia is under translational control. J. Biol. Chem., 261:16126-16132, (1986).
2. Katze, M.G., DeCorato, D., Safer, B., Galabru, J., and Hovanessian, A.G.: Adenovirus VAI RNA complexes with the 68,000 M<sub>r</sub> protein kinase to regulate its autophosphorylation and activity. EMBO 6:689-697, 1987.
3. Jagus, R. and Safer, B.: Second phosphorylation site on the alpha subunit of eIF-2 in rabbit reticulocyte lysate. Adv. in Cyclic Nucl. Res. and Protein Phosphorylation, in press, 1987.
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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02216 08 MH

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Correction of Genetic Defects by Gene Transfer

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W. French Anderson, Chief, LMH, NHLBI  
Philip Kantoff, Med. Staff Fellow, LMH, NHLBI  
Daniel Kuebbing, Sen. Staff Fellow, LMH, NHLBI  
Martin Eglitis, Sen. Staff Fellow, LMH, NHLBI  
Jeanne McLachlin, Visiting Assoc., LMH, NHLBI  
Judith DiPietro, Biologist, LMH, NHLBI  
Sheri Bernstein, Biologist, LMH, NHLBI  
Robert Moen, Med. Staff Fellow, LMH, NHLBI

Scott Freeman, Visiting Fellow, LMH, NHLBI  
Ken Cornetta, Visiting Fellow, LMH, NHLBI  
Jamie Zwiebel, MSF, LMH, NHLBI  
Evelyn Karson, MSF, LMH, NHLBI  
Robert Weider, MSF, LMH, NHLBI  
Jane Selegue, Biologist, LMH, NHLBI

COOPERATING UNITS (if any) A. Nienhuis, CHB, NHLBI; E. Gilboa, Sloan-Kettering Cancer Center NY; M. Blaese, MET, NCI; R. O'Reilly, Sloan-Kettering Cancer Center, N.Y.; E. Zanjani, VA Hospital, Minn. MN; M. Harrison, U. of California, Medical School, San Francisco, CA

## LAB/BRANCH

Laboratory of Molecular Hematology

## SECTION

Section on Molecular Genetics

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

11.2

8.4

2.8

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A highly efficient procedure for transferring functional genes into mammalian cells has been developed using retroviral vectors as a delivery system. When mouse bone marrow cells are infected in vitro with a NeoR gene and reinjected into a lethally irradiated recipient mouse, 85-90% of the stem cells (CFU-S) can be shown to carry an intact copy of the NeoR gene. The majority of these cells produce the NeoR gene product: phosphotransferase. Retroviral vectors containing the human gene for the enzyme adenosine deaminase (ADA) as well as the NeoR gene have been made. Using the knowledge obtained from the murine system a non-human primate autologous bone marrow transplantation/gene transfer protocol has been developed. Low levels of both the human ADA gene and the NeoR gene have been expressed in the peripheral blood cells of sever monkeys. In utero gene transfer has also been successfully accomplished using a fetal sheep marrow transplantation protocol. In addition, these vectors have been used to introduce exogenous genes into human bone marrow progenitors in vitro. These studies are preliminary to attempting human gene therapy in patients suffering from ADA severe combined immunodeficiency disease.

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Project Description:

Objectives: The objective of this project is to develop methods for transferring functional genes into mammalian tissue culture cells and into intact animals. The techniques, when ready, would be used for attempting to correct genetic diseases in human patients.

Methods Employed:

1. Tissue culture cells are grown under standard tissue culture conditions.
2. Plasmids containing specific cloned genes are made by standard recombinant DNA techniques.
3. Gene transfer by retroviral vectors uses standard retroviral techniques for infection and analysis.
4. Genomic mapping is carried out by standard recombinant DNA techniques.
5. Bone marrow transplantation into lethally irradiated mice and primates is by published procedures.
6. In utero bone marrow transplantation into fetal sheep is by published procedures.
7. Colony assays (CFU-C, BFU-E, CFU-E) are by published procedures.

Major Findings:

1. The human ADA gene (as well as the Neo<sup>R</sup> NPT gene) has been efficiently expressed in approximately 0.5% of the circulating mononuclear cells of one monkey and at lower levels in several other animals. A primate bone marrow transplantation (BMT) program has been established in two locations: Memorial Sloan Kettering Institute where the primate used is the Cynomolgus macaque, and at the National Institutes of Health where the primate used is the rhesus monkey. The two main vectors used in these studies are N2 and SAX. Neo and ADA represent the principal genes delivered by the N2 and SAX vectors respectively. The method of infection of bone marrow (BM) cells was either by cocultivation of the cells with virus producing cells or by virus-containing supernatant infection. Reconstitution represented the full recovery of all lineages. Monkey 3 (Bill), despite not achieving full reconstitution, was DNA positive, weakly NPT positive and very weakly human ADA positive at the time of death. The supernatant infection protocol was replaced by cocultivation protocol beginning with monkeys 9 and 10 (Lucy and Ethel). Ethel was DNA and NPT negative but like Bill was very weakly human ADA positive. After improvements in the supernatant infection protocol, monkeys 11 and 12 (Robert and Kyle) demonstrated substantially higher levels of human ADA gene activity. Fractionation by FPLC column chromatography of Robert's 69 day post-transplant (Ficoll-processed) peripheral blood demonstrated that conversion activity (of adenosine to inosine) in Robert reached 66% in the peak human fractions. Overall the amount of human ADA produced by Robert represented about 0.5% of the endogenous monkey ADA activity. Independent verification that vector DNA was present and expressing in Robert's blood was obtained by in situ



hybridization. Using a NeoR specific gene probe, approximately 1 cell in 200 was NeoR RNA positive.

2. In utero gene transfer and expression have been demonstrated in the fetal lamb. To test the feasibility of fetal BMT/gene transfer, an in utero sheep gene transfer protocol was established. Circulating hematopoietic cells from a 96 day old fetal lamb were removed by exchange transfusion, infected with the N2 vector by the supernatant infection protocol, and reinfused back into the lamb. The fetal lamb was allowed to grow and was born normally at term (145 days gestation). One week after birth bone marrow cells from the lamb were evaluated in colony assays in the presence or absence of G418. However G418<sup>R</sup> colonies were obtained in the infected animal (CFU-C - 22%, CFU-MIX - 39%, BFU-E - 35%, CFU-e - 22%) representing successful gene transfer and expression of the NeoR gene 62 days after transplantation. Although the proportion of G418<sup>R</sup> colonies recovered declined somewhat between 5 and 10% remained drug resistant several months after birth.

3. Human hematopoietic progenitor cells can be infected with the vector SAX and are resistant to G418 in a CFU-C assay at an efficiency of 1-2%. Normal human bone marrow was used as a source of progenitor cells to test for the efficiency of retroviral infection. In several different experiments, BM cells were infected with the N2 virus either by a cocultivation infection protocol or a supernatant infection protocol and plated in methylcellulose in the presence or absence of G418. Each colony growing in the presence of G418 represents functional gene transfer in a progenitor cell. The number of colonies surviving treatment with virus divided by the number surviving no treatment indicates the fraction viability of progenitor cells carrying an expressing NeoR gene. The viability of progenitor cells during the procedure was high (CFU-E = 32%, BFU-E = 115%, CFU-GM = 102%). The level of functional gene transfer into all 3 progenitor cells was 1-2%. In like manner, bone marrow cells from patients with ADA deficiency can also be infected with the SAX vector. These genetically defective cells also were shown to express the NeoR gene of the SAX vector in 1-2% of the CFU-C progenitor cells.

#### Significance to Biomedical Research and the Program of the Institute:

The long-term aim of much of the work in molecular genetics is to develop techniques for treating or curing human genetic defects. This project utilizes recombinant DNA technology, retrovirology, mouse genetics, embryology, and cell biology techniques to try to accomplish this goal.

#### Proposed Course of the Project:

The primate and murine bone marrow transplant model systems will continue to be developed in order to ensure that ADA can be expressed efficiently in the bone marrow and blood cells of long-term reconstituted animals. The safety of the procedure will be analyzed in detail in preparation for carrying out the same procedure in patients with ADA-SCID.





1. Kantoff, P.W., Kohn, D.B., Mitsuya, H., Armentano, D., Sieberg, M., Zwiebel, J.A., Eglitis, M.A., McLachlin, J.R., Wiginton, D.A., Hutton, J.J., Horowitz, S.D., Gilboa, E., Blaese, R.M., and Anderson, W.F.: Correction of adenosine deaminase deficiency in human T and B cells using retroviral-mediated gene transfer. *Proc. Natl. Acad. Sci. USA*, 83, 6563-6567, 1986.
2. Goldberg, S.Z., Kuebbing, D., Trauber, D., Schafer, M., Lewis, S., Popp, R., Anderson, W.F.: A 66-base pair insert bridges the deletion responsible for a mouse model of  $\beta$ -thalassemia. *J. Biol. Chem.*, 261, 12368-12374, 1986.
3. Anderson, W.F.: Prospects for human gene therapy in the born and unborn patient. *Clin. Obst. Gyn.* 29, 586-594, 1986.
4. Curcio, M.J., Kantoff, P.W., Schafer, M., Anderson, W.F., Safer, B.: Compensatory increase in levels of  $\beta$  minor globin in murine -thalassemia is under translational control. *J. Biol. Chem.* 261, 16126-16132, 1986.
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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02217 02 MH

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Gene Expression Utilizing Nucleic Acid Manipulations

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J.A. Thompson, Expert, LMH, NHLBI R. Cohen, Med. Staff Med. LMH, NHLBI  
Others: B. Safer, Medical Officer, LMH, NHLBI K. Anderson, Guest Worker, LMH, NHLBI  
S. Garfinkel, Bio. Lab. Tech., LMH, NHLBI F. Anderson, Chief, LMH, NHLBI  
J. Zwiebel, Sen. Staff Fell., LMH, NHLBI  
D. Kuebbing, Sen. Staff Fell., LMH, NHLBI  
S. Freeman, Guest Worker, LMH, NHLBI  
D. Meunchau, Med. Tech., LMH, NHLBI  
J. DiPietro, Res. Biol., LMH, NHLBI

COOPERATING UNITS (if any) R. Blakesely, Life Technologies, Inc., Gaithersburg, MD; Robert Wells, U. of AL, Birmingham, AL; L. Reid, Albert Einstein College of Medicine, Bronx, N.Y.; G. Zon, Applied Biosystems, Inc., Foster City, CA.; T. Brendler, PRI Frederick, MD.; and T. Maciag, American Red Cross, Rockville, MD.

## LAB/BRANCH

Laboratory of Molecular Hematology

## SECTION

Section on Molecular Cloning

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.2

## PROFESSIONAL:

1.3

## OTHER:

1.9

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The nature and position of transcriptional control elements responsible for the differential control of gene expression in eukaryotic cells have not been precisely defined. A primary goal of this section is to develop new methods to investigate transcriptional control mechanisms, which are mediated by nucleic acid promoter elements, utilizing an active in vitro transcription system. Tissue-specific promoter elements will be used to stimulate gene expression in vivo utilizing retroviruses as mediators of specific gene transfer.

To identify components required for transcription of mRNA by RNA polymerase II, distinct transcriptional complexes have been characterized using newly developed techniques of DNA competition and have been purified using newly developed techniques of DNA affinity chromatography. Proteins associated with specific DNA sequences following affinity purification are being used to identify: 1) Transcription factors and their functionality and 2) DNA control sequences and topology necessary for regulated initiation and elongation of in vitro transcriptional intermediates.

Specific synthetic control sequences of DNA are being assembled, based on the presence of tissue-specific transcription factors and nucleic acid topology. These synthetic promoters are being used to stimulate expression and secretion of the genes for adenosine deaminase, rat growth hormone and ECGF in endothelial, and liver cells, respectively, following cloning into retroviruses. Both in vitro and in vivo infections with these modified expression vectors are being studied as models of gene transfer into genetically deficient cells.





Project Description:

Objectives: The major goals are: 1) to determine specific DNA sequences and structures responsible for transcriptional control of gene expression; 2) to develop new methods for identifying, purifying and characterizing protein factors involved in transcriptional regulation; 3) to construct nucleic acid sequences for tissue-specific insertion, expression and secretion of synthetic genes; and 4) to design a biosystem which will facilitate the growth in tissue culture and in vivo of primary hepatocytes.

Methods Employed: Conventional liquid chromatography and HPLC utilizing principles of gel-exclusion, ion-exchange, affinity partitioning, reverse-phase and ion-pairing have been employed for rapid purification of both proteins and nucleic acids involved in transcription. Active transcription systems have been developed for K-562, HeLa and hepatocyte cells using non-disruptive procedures (perfusion, sucrose gradients, etc.) which maintain cell ultrastructure. Partially hepatectomized livers will be used to obtain large amounts of transcriptionally active hepatocytes for identifying transcription factors and promoter structure. DNA promoter sequences are identified by binding of radiolabeled DNA-protein complex to nitrocellulose filters; transcriptional factors recognizing these specific promoter sequences are identified by binding of radiolabeled DNA fragments to electrophoretically resolved proteins immobilized on a solid support (southwestern blotting) or by isolation of discrete DNA protein complexes on low ionic strength polyacrylamide. Binding sites are mapped by DNase I and dimethylsulfate footprinting. Specific DNA-binding peptides are affinity labeled using synthetic radiolabeled DNA sequences. Synthesis of oligonucleotide probes, linkers and promoter control elements are generated using automated solid phase phosphoramidite chemistry. Covalent modification of these DNA fragments are employed using conventional methods of organic chemistry. Urea-PAGE, SDS-PAGE, and agarose gel electrophoresis are used to analyze DNA fragments, proteins and RNA generated from transcriptionally active extracts. Specific sequences of RNA are measured by hybridization to specific nucleic acid probes followed by RNase T1 analysis using urea-PAGE. Plasmids containing specific nucleic acid sequences are generated using conventional cloning methods in molecular biology: restriction enzymology, DNA modifying enzymes (kinase, ligase, etc.), site-specific mutagenesis, oligonucleotide synthesis, DNA sequencing and transformation and growth into E. coli cells. Genomic libraries are generated in a manner similar to construction of plasmids and are identified by dot blot and southern analysis using standard principles of hybridization. Primary hepatocytes are grown in tissue culture using various biomatrices generated biologically (cloned differentiated rat hepatocytes) or extracted (Type IV collagen from EHS murine embryonal carcinoma). Hepatocytes are infected with retroviruses carrying specific genetic markers either in tissue culture or surgically implanted into genetically deficient rats. Gene transfer and expression are assayed by northern analysis of mRNA, Southern analysis of DNA, enzymatic activity of expressed protein, resistance to cytotoxic chemicals and in situ hybridization of specific nucleic acid sequences. Computer modeling and analysis is used to help generate specific sequences of DNA promoters for gene expression.



sequences, and DNA topology involved in regulating activity.

8. Both primary and cloned rat hepatocytes have been infected with retroviruses containing the gene for Neo. Following treatment with G418, uninfected cells were killed, whereas infected cells continued growth. DNA and RNA was extracted and assayed for specific sequences. Phosphotransferase activity was analyzed and in situ hybridizations performed to confirm gene transfer.

9. Infected rat hepatocytes have been implanted into genetically deficient animals and remained viable for up to two weeks.

#### Proposed Course of the Project:

1. Polypeptides identified as components of transcriptional complexes will be purified and characterized as to specific function especially in relation to DNA structure.

2. In vitro transcription using hepatocytes perfused from normal and partially hepatectomized livers as well as primary hepatocytes from tissue culture will continue to be developed.

3. Different promoter activity, including synthetic, will be compared in liver extracts to determine various aspects of regulatory mechanisms.

4. Purification of identified transcriptional factors by affinity chromatography will be followed by antibody production, peptide sequencing, generation of mixed probes and screening of genomic libraries to characterize specific gene sequences of these proteins.

5. Development of synthetic tissue-specific promoter cassettes for enhanced gene expression following retroviral transfer primarily into primary hepatocytes will be continued.

6. In vivo implants (or infections) of rat hepatocytes will continue to be developed in genetically deficient animals for studying gene transfer and regulation of transcription.

#### Publications

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2. Thompson, J.A.: A review of high performance liquid chromatography in nucleic acids research. IV. Isolation, purification, and analysis of DNA restriction fragments. *BioChromatography* 2:4-18 (1987).
3. Thompson, J.A.: A review of high performance liquid chromatography in nucleic acids research. V. Nucleic acid affinity techniques in DNA and RNA research. *BioChromatography* 2:68-78 (1987).
4. Safer, B., Cohen, R.B., Garfinkel, S. and Thompson, J.A.: DNA affinity labeling of adenovirus-2 upstream promoter sequence binding factors identifies two distinct proteins. *Mol. Cell Biol.* Submitted (1987).



Annual Report of the  
Section on Laboratory Animal Medicine and Surgery, Surgery Branch  
Division of Intramural Research  
National Heart, Lung, and Blood Institute  
October 1, 1986 to September 30, 1987

The Section functions primarily in a support role to all laboratories of IR providing care for many species of animals, technical assistance in preparation and maintenance of animal models for various experimental regimens, and the development of animal resources not otherwise available.

Maintenance of various small animal species has been accomplished in designated areas in close proximity to IR laboratories in Buildings 3, 10, and 36. Large animal species were maintained in Buildings 3, 28, the NIHAC, and at Luray, Virginia. Postoperative intensive care and treatment of surgery patients was completed in Buildings 3, 14-E, and 28. However, animal holding has been phased out of Building 3 during the report period.

The animal surgery laboratory located in Building 14-E supported studies for investigative staff in the Cardiology Branch, Clinical Hematology Branch, Laboratory of Kidney and Electrolyte Metabolism, Pulmonary Branch, Laboratory of Technical Development, and the Surgery Branch in preparation of experimental animal models, completing cardiovascular studies and in collecting various biological specimens. The laboratory operates an x-ray catheterization suite, blood analysis laboratory, sterile operating suites, and special study suites required to meet requirements.

The NHLBI sheep colony was phased out during this report period. A contract was awarded to Environmental Diagnostics, Burlington, NC to meet future supply requirements.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03402-11 LAMS

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

NHLBI Laboratory Sheep Colony

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Joseph E. Pierce, D.V.M., Chief, SLAMS, SB, DIR, NHLBI

## COOPERATING UNITS (if any)

1. VRB, DRS

## LAB/BRANCH

Office of the Director of Intramural Research

## SECTION

Section on Laboratory Animal Medicine and Surgery

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Laboratory Sheep Colony was an NIH animal resource providing varied age sheep that met specific year-round requirements of the Laboratory of Kidney and Electrolyte Metabolism, Pulmonary Branch, Laboratory of Technical Development and the Surgery Branch, DIR, NHLBI.

Practices that have contributed to reduction of undesired seasonal variables include: (1) continuous prophylactic immunization of all age animal groups; (2) accurate pregnancy diagnosis during first trimester using Doppler ultrasound; (3) monitoring of animal health using various diagnostic laboratory techniques; and (4) many husbandry techniques unique to this colony. Such practices have been cost prohibitive in commercial sheep flocks that result in inconsistent availability and existence of varied states of health in animals delivered for laboratory use.

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Project Description:

The breeding colony was phased out during the report period.

Contract Information

Contract Number: 263-80-C0007 - approximately \$44,575 - 10/1/86-12/31/86

Contract Site: White House Farms, Inc.  
Rt. 1, Box 403-E  
Luray, Virginia 22835

PI: Max Foltz, Contractor  
Rick Miller, Colony Manager

Total Man Years: 1.25

Professional: .5

Other: .75

664



Annual Report of the Pathology Branch  
Division of Intramural Research  
National Heart, Lung, and Blood Institute  
October 1, 1986, to September 30, 1987

As in past years, studies focused on various types of cardiovascular diseases including coronary, valvular, myocardial, and miscellaneous varieties.

CORONARY ARTERY DISEASE

Cardiac weight at necropsy was examined in 200 patients who had coronary artery bypass grafting (CABG) for angina pectoris, and its relation to early (<60 days) and late (>60 days) death after CABG was sought. One hundred and sixteen patients dying early had a lower mean heart weight than did the 84 patients dying late ( $443 \pm 94$  g vs  $494 \pm 107$  g;  $p < .01$ ). The relation of heart weight to early or late death occurred with each sex. The mean heart weight of the 81 men dying early was less than that of the 72 men dying late ( $471$  g vs  $503$  g;  $p < .05$ ), and the mean heart weight of the 35 women dying early was less than that of the 12 women dying late ( $379$  g vs  $444$  g;  $p < .05$ ). Most patients with hearts of normal weight were in the early death group: of the 16 women with hearts of normal weight ( $\leq 350$  g), 15 (94%) died early, and of the 34 men with hearts of normal weight ( $\leq 400$  g), 21 (62%) died early. Conversely, most patients in the late death group had hearts of increased weight: of the 12 women dying late, 11 (92%) had hearts of increased weight, and of the 84 men dying late, 70 (93%) had hearts of increased weight. The mean cardiac weights of the 118 patients with left ventricular scars was larger than that of the 82 patients without scars ( $490$  g vs  $429$  g;  $p < .01$ ), and these differences were observed within each sex and in both early and late death groups. This study suggests that patients with normal or near normal sized hearts have a higher mortality early after CABG than do persons having hearts of increased weight.

The internal mammary artery in recent years has proved to have better long-term patency than the saphenous vein when used as an aortocoronary conduit. We studied internal mammary arteries and saphenous veins used as conduits for 1 day, 4 months, 8 years and 12 years, respectively, in 4 patients. At necropsy, none of the internal mammary arteries had any degree of narrowing. In contrast, the saphenous veins used as conduits in the same patient contained considerable amounts of atherosclerotic plaque. Additionally, the internal mammary arteries were much smaller than were the saphenous veins, suggesting that they did not change in size during the period when used as a conduit, whereas the saphenous veins got larger despite the fact that they developed a great deal of atherosclerotic plaque. This study supports the view obtained clinically that internal mammary arteries are better conduits for CABG than are saphenous veins.

Spasm of native epicardial coronary arteries is a well known occurrence and several surgeons have observed spasm in a saphenous vein used as an aorto-coronary bypass. We studied a patient who had CABG, a myocardial infarct postoperatively, and saphenous veins used as aorto-coronary conduits were virtually obliterated by spasm of the conduit, providing anatomic confirmation of a physiologic event.



Clinical and necropsy findings were described in 21 patients aged 43 to 78 years (mean 63) (19 men [90%]) who had grossly visible calcified myocardial infarcts. The interval from the first clinically apparent acute myocardial infarct to death (20 patients) ranged from 2 to 26 years (mean 12). This interval was >5 years in 17 patients (85%) and >10 years in 11 patients (55%). The ages at the first clinically apparent acute myocardial infarct ranged from 36 to 72 years (mean 51). Of the 21 patients, 17 (81%) had clinical evidence of chronic congestive heart failure; 12 (57%) had left ventricular aneurysms; 8 (38%) had documented episodes of ventricular tachycardia, and 5 had angina pectoris. At necropsy, the heart weight was increased (>400 g) in all (mean 557 g), the left ventricular cavity was dilated in all, and at least 1 and usually 2 or 3 (86%) major epicardial coronary arteries were narrowed >75% in cross-sectional-area by atherosclerotic plaque. Thus, patients with calcified myocardial infarcts are usually men, they usually have the myocardial infarct which calcifies at a relatively young age, the calcified wall commonly is part of an aneurysmal wall, the left ventricular cavity is virtually always dilated, the heart weight is increased, and progressive congestive heart failure is the usual mode of death.

Certain cardiac morphologic findings were described in 16 necropsy patients having operative closure of an acquired ventricular septal defect (VSD) during acute myocardial infarction (AMI). Of the 16 patients, 6 were women (mean age  $69 \pm 7$  years) and 10 were men (mean age  $60 \pm 11$  years). The AMI associated with the VSD was the first coronary event in 13 patients (81%). The median intervals from the onset of the AMI to death was 11 days, and from the onset of the AMI to operative closure of the VSD, 14 days. Eight patients died in the operating room or within 2 hours of operation. Coronary artery bypass grafting was performed simultaneously with the VSD closure in 7 patients. Death was attributed to unsuccessful VSD closure in 5 patients, to inadequate left ventricular cavity after resection of necrotic myocardium in 5 patients, and to inadequate viable left ventricular myocardium in 4 patients. Heart weights were increased in 14 patients (88%). The AMI associated with the VSD was anterior in 9 patients and posterior (inferior) in 7.

Combined rupture of the left ventricular free wall and either ventricular septum or papillary muscle is rare and this possibility has received little attention. We described certain clinical and necropsy findings in 7 patients who had combined rupture of both free wall and septum during AMI. Of variables analyzed no significant differences were observed from those in patients with isolated rupture of either free wall or ventricular septum.

#### VALVULAR HEART DISEASE

Certain clinical and mitral valvular morphologic findings were described in 83 patients (aged 26-79 years [mean 60]; 26 women [31%] and 57 men [69%]) with mitral valve prolapse (MVP) and mitral regurgitation (MR) severe enough to warrant mitral valve replacement. All 83 operatively excised valves were examined by the same person and all excised valves had been purely regurgitant (no element of stenosis). No patient had hemodynamic evidence of dysfunction of the aortic valve. In each valve a portion of posterior mitral leaflet was elongated such that the distance from distal margin to basal attachment of this leaflet was similar to the distance from distal margin of anterior



leaflet to its basal attachment to left atrial wall. Two major mechanisms for the severe MR were found: 1) dilatation of the mitral annulus with or without rupture of chordae tendineae, and 2) rupture of chordae tendineae with or without dilatation of the mitral annulus. Of the 83 patients, 48 (58%) had both dilated annuli (>11 cm in circumference) and ruptured chordae tendineae; 16 (19%) had dilated annuli without ruptured chordae, and 16 (19%) had ruptured chordae without significant annular dilatation. In 3 patients neither the annulus was dilated nor were chordae ruptured, and therefore the mechanism of the MR in them is uncertain. Mitral chordal rupture was nearly as frequent in the 64 patients with clearly dilated annular circumferences as in the 19 patients with normal or insignificantly dilated annular circumferences ( $\leq 11$  cm.).

### CARDIOMYOPATHY

Certain clinical and cardiac necropsy findings in 152 patients aged 16 to 78 years (mean 45) with idiopathic dilated cardiomyopathy (IDC) were described: 109 (72%) were men and 43 (28%) were women. Compared to the women, the men had a significantly ( $p < .05$ ) shorter mean duration of chronic congestive heart failure (CHF) (43 months - vs - 69 months), a higher percentage of habitual alcoholism (40% - vs - 24%), and a higher mean heart weight (632 g - vs - 551 g). The male-to-female ratio among the 58 known alcoholics was 7.3:1 and among the 70 known non-alcoholics, 1.5:1 ( $p < .05$ ). The mean duration of clinical evidence of CHF was similar among the known alcoholics and the known non-alcoholics (each 50 months). Of the 152 patients, 148 (97%) had clinical evidence of chronic CHF; in 114 patients it was the initial manifestation of IDC, and in most it became intractable and caused death. The interval from onset of chronic CHF to death (known in 120 patients) ranged from 1 to 264 months (mean 54). Comparison of the 27 patients surviving >72 months after onset of chronic CHF to the 64 patients surviving  $\leq 36$  months disclosed a significantly higher frequency in the longer survival group of older patients, of women, of habitual alcoholics, of patients with chest pain syndromes, diabetes mellitus, pulmonary emboli, of patients treated with warfarin, and of patients with bigger hearts at necropsy. Each of the 4 patients without chronic CHF died suddenly and sudden death was the initial manifestation of IDC in them. An additional 33 patients also died suddenly but each of them previously had had chronic CHF. Of the 79 patients (of 131 where information available) with either pulmonary or systemic emboli or both, 67 (85%) had either right- or left-sided thrombi or mural endocardial plaques or both, whereas of the 52 patients without emboli, 36 (69%) had intracardiac thrombi or plaques ( $p < .05$ ). Electrocardograms in the last 6 months of life in 101 patients disclosed atrial fibrillation in 25; complete left (41 patients) or right (6 patients) or indeterminate (4 patients) bundle branch block in 51 patients; QRS voltage indicative of ventricular hypertrophy in 44 patients (left in 39 patients). The patients with compared to those without left bundle branch block had a significantly longer duration of CHF (83 months - vs - 43 months), larger mean heart weights (628 g - vs - 590 g), and a higher frequency of grossly visible scars (44% - vs - 13%). The total 12-lead QRS amplitude in 35 men ranged from 74 to 250 mm (mean 147) (10 mm = 1 mV) and in the 14 women, from 75 to 243 mm (mean 167). The hearts at necropsy in the women ranged from 360 to 860 g (mean 551) and in the men, 400 to 940 g (mean 632) ( $p < .05$ ). Grossly visible left ventricular





scars were observed in 22 patients (14%). Histologic examination (96 patients) of sections of the left ventricular walls disclosed interstitial or replacement fibrosis in 55 patients (57%), and inflammatory-cell infiltrates in only 5 patients.

Controversy exists over the role of endomyocardial biopsy in evaluating patients with IDC, particularly in regard to detecting myocarditis and in assessing prognosis. Interobserver variability, if high, could explain conflicting reports. To assess for this, biopsy specimens from 16 patients with the clinical diagnosis of IDC were submitted to 7 cardiac pathologists. The same slides were independently reviewed by each, and assessed for fibrosis, hypertrophy, nuclear changes, mean lymphocyte count per high power field, and presence or absence of myocarditis. The prevalence of significant fibrosis ranged from 25% to 69%; of hypertrophy, from 19% to 88%; of nuclear changes, from 31% to 94%, and of abnormal lymphocyte count, from 0 to 38%. One or more pathologists diagnosed definite or possible myocarditis in 11 of the 16 patients. It was concluded from the study that interobserver variability is high in interpreting biopsy specimens from patients with IDC and that quantitative and standardized methods are needed to increase diagnostic consistency.

To determine the frequency and significance of mitral anular calcium (MAC) in patients with hypertrophic cardiomyopathy (HC), 43 clinical and morphologic variables in 200 necropsy cases of HC were examined. Of 100 patients  $\leq$  40 years of age, none had MAC. Of the 100 necropsy patients  $>$  40 years of age, 30 (30%) had MAC, and of them 21 (70%) were women. The mean age of the 30 MAC patients was older than that of the 70 non-MAC patients  $>$  40 years of age (66 years-vs.-53 years). The frequency of MAC increased with age. MAC was present in 3 (10%) of 31 patients aged 41 to 50 years; in 6 (18%) of 34 patients aged 51 to 60 years, in 11 (52%) of 21 patients aged 61 to 70 years, and in 10 (71%) of 14 patients aged 71 to 90 years. Compared to the 70 patients  $>$  40 years of age without MAC, the 30 patients  $>$  40 years of age with MAC had higher average systemic arterial peak systolic pressures (133 mm Hg - vs -113 mm Hg); a higher percent of the MAC patients had calcific deposits in the epicardial coronary arteries (93% - vs - 41%) and in the aortic valve cusps (33% - vs - 6%), and a higher percent of the MAC patients had severe narrowing by atherosclerotic plaques of 1 or more of the 4 major epicardial coronary arteries (47% - vs - 24%). The frequency of a history of systemic hypertension, diabetes mellitus and total serum cholesterol levels  $>$ 200 mg/dl in the patients with and without MAC was similar. In no patient with MAC by hemodynamic studies was there evidence of obstruction to left ventricular inflow. The frequency of mitral regurgitation by left ventricular angiography was similar in the patients with and without MAC.

The prevalence of myocarditis and dilated cardiomyopathy in 71 consecutive necropsy patients with the Acquired Immunodeficiency Syndrome (AIDS) was evaluated: myocarditis was found in 37 cases (52%) and was mostly mononuclear, mild and patchy in distribution, including 7 (10%) cases with features consistent with dilated cardiomyopathy. Dilated cardiomyopathy at necropsy was associated with major cardiac dysfunction, pericardial effusion and increased mean heart weight (379 g vs. 320 g,  $p < 0.05$ ) compared to the other 64 AIDS patients. Although viral, protozoan, bacterial, fungal and



mycobacterial or opportunistic pathogens were present in myocardial sections of 7 of 37 myocarditis cases, these included only 2 cases with dilated cardiomyopathy. Therefore, the etiology of myocarditis in most AIDS patients remains uncertain. The causes of death in 45 cases (62%) was pulmonary failure, and cardiovascular death in 6 cases (8%), 4 of whom had dilated cardiomyopathy. Thus, myocarditis is a frequent necropsy finding in patients with AIDS and it may cause dilated cardiomyopathy and cardiac failure.

#### MISCELLANEOUS INVESTIGATIONS

We and others have noted the coronary sinus (CS) to be enlarged in some patients with chronic congestive heart failure and in virtually all patients with drainage of a left superior vena cava into the CS. The CS is receiving increasing attention because of its potential usefulness as a route for retrograde delivery to ventricular myocardium of cardioplegic solutions during cardiopulmonary bypass, of various antiarrhythmic agents during electrophysiologic testing, and of blood and blood substitutes for symptomatic myocardial ischemia. In 1984, an entire symposium was devoted to myocardial protection via the CS, and the proceedings from it were published. Despite the increasing interest in the CS, little anatomic information is available in subjects with normal hearts and in patients with abnormal hearts. To fill this void, we measured the circumference of the CS in 50 normal human hearts and in 234 hearts with various cardiac diseases unassociated with intercirculatory shunts. The mean CS circumference (in mm) in both men and women in the normal hearts ( $19 \pm 3$ ) and those with systemic hypertension ( $22 \pm 3$ ), aortic stenosis ( $21 \pm 3$ ), hypertrophic cardiomyopathy ( $21 \pm 4$ ) and cor pulmonale ( $23 \pm 2$ ) were similar. The mean circumferences of the CS in the chronic hemodialysis ( $30 \pm 5$ ), ischemic cardiomyopathy ( $32 \pm 5$ ) and idiopathic dilated cardiomyopathy ( $36 \pm 6$ ) groups all differed significantly from that of the normal hearts.

The types of and quantities of cardiological publications in the USA have changed drastically in recent years and these changes were analyzed. The small independent medical publisher in the USA is vanishing. Medical publishers in the USA today primarily are parts of larger non-medical publishing corporations, and the non-medical publications are altering the medical publications. The "throw-away" journals are increasing and their existence is diminishing the reading time available and advertising support provided to the peer-review subscription journals. The publication of symposia proceedings supported entirely by grants from single pharmaceutical or medical device companies in otherwise peer-review journals is increasing. It is better for physicians not to exert efforts to discontinue this practice, which is here to stay, but to exert their influence to reduce excess bias and overstatement. The peer-review subscription journals, most of which are published by for-profit corporations, are the sources of our new medical information and the "storehouse" of our knowledge. They need our strong support. They need to be published on acid-free paper which will not deteriorate. We need more editorial pages in the peer-review subscription journals so that more review and experimental type articles can be published, and less pages in the "throw-away" journals which diminish support available to the peer-review subscription journals.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03900-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Calcification of healed myocardial infarcts.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others: R. Kaufman\*

\*High School Student

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Clinical and necropsy findings are described in 21 patients aged 43 to 78 years (mean 63) (19 men [90%]) who had grossly visible calcified myocardial infarcts. The interval from the first clinically apparent acute myocardial infarct to death (20 patients) ranged from 2 to 26 years (mean 12). This interval was >5 years in 17 patients (85%) and >10 years in 11 patients (55%). At necropsy, the heart weight was increased (>400 g) in all (mean 557 g), the left ventricular cavity was dilated in all, and at least 1 and usually 2 or 3 (86%) major epicardial coronary arteries were narrowed >75% in cross-sectional area by atherosclerotic plaque.



Gross Description:

Clinical and necropsy findings are described in 21 patients aged 43 to 78 years (mean 63) (19 men [90%]) who had grossly visible calcified myocardial infarcts. The interval from the first clinically apparent acute myocardial infarct to death (20 patients) ranged from 2 to 26 years (mean 12). This interval was >5 years in 17 patients (85%) and >10 years in 11 patients (55%). At necropsy, the heart weight was increased (>400 g) in all (mean 557 g), the left ventricular cavity was dilated in all, and at least 1 and usually 2 or 3 (86%) major epicardial coronary arteries were narrowed >75% in cross-sectional area by atherosclerotic plaque.

The ages at the first clinically apparent acute myocardial infarct ranged from 36 to 72 years (mean 51). Of the 21 patients, 17 (81%) had clinical evidence of chronic congestive heart failure; 12 (57%) had left ventricular aneurysms; 8 (38%) had documented episodes of ventricular tachycardia, and 5 had angina pectoris. Thus, patients with calcified myocardial infarcts are usually men, they usually have the myocardial infarct which calcifies at a relatively young age, the calcified wall commonly is part of an aneurysmal wall, the left ventricular cavity is virtually always dilated, the heart weight is increased, and progressively congestive heart failure is the usual mode of death.

Publication:

Roberts WC, Kaufman, RJ\*. Calcification of healed myocardial infarcts. Am J Cardiol 1987; 60:28-32.

\*High School Student, Silver Spring, Maryland





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03901-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cardiac weight and its prognostic significance after coronary artery bypass

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others: J.M. Kalan, MD

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Cardiac weight at necropsy was examined in 200 patients who had coronary artery bypass grafting (CABG) for angina pectoris, and its relation to early (<60 days) and late (>60 days) death after CABG was sought. The 116 patients dying early had a lower mean heart weight than did the 84 patients dying late ( $443 \pm 94$  g vs  $494 \pm 107$  g;  $p < .01$ ). The relation of heart weight to early or late death occurred with each sex. Most patients with hearts of normal weight were in the early death group: of the 16 women with hearts of normal weight ( $\leq 350$  g), 15 (94%) died early, and of the 34 men with hearts of normal weight ( $\leq 400$  g), 21 (62%) died early. Conversely, most patients in the late death group had hearts of increased weight, and of the 84 men dying late, 70 (93%) had hearts of increased weight. This study suggests that patients with normal or near normal sized hearts have a higher mortality early after CABG than do persons having hearts of increased weight.



Gross Description:

Cardiac weight at necropsy was examined in 200 patients who had coronary artery bypass grafting (CABG) for angina pectoris, and its relation to early (<60 days) and late (>60 days) death after CABG was sought. The 116 patients dying early had a lower mean heart weight than did the 84 patients dying late ( $443 \pm 94$  g vs  $494 \pm 107$  g;  $p < .01$ ). The relation of heart weight to early or late death occurred with each sex. Most patients with hearts of normal weight were in the early death group: of the 16 women with hearts of normal weight ( $\leq 350$  g), 15 (94%) died early, and of the 34 men with hearts of normal weight ( $\leq 400$  g), 21 (62%) died early. Conversely, most patients in the late death group had hearts of increased weight, and of the 84 men dying late, 70 (93%) had hearts of increased weight. This study suggests that patients with normal or near normal sized hearts have a higher mortality early after CABG than do persons having hearts of increased weight.

The mean heart weight of the 81 men dying early was less than that of the 72 men dying late (471 g vs 503 g;  $p < .05$ ), and the mean heart weight of the 35 women dying early was less than that of the 12 women dying late (379 g vs 444 g;  $p < .05$ ). The mean cardiac weights of the 118 patients with left ventricular scars was larger than that of the 82 patients without scars (490 g vs 429 g;  $p < .01$ ), and these differences were observed within each sex and in both early and late death groups.

Publication:

Kalan JM, Roberts WC. Cardiac weight and its prognostic significance after coronary artery bypass grafting for angina pectoris. New Eng J Med 1987; (In Press).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03902-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cardiac morphologic observations after operative closure of acquired ventricular

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others: J.M. Mann, MD

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Certain cardiac morphologic findings are described in 16 necropsy patients having operative closure of an acquired ventricular septal defect (VSD) during acute myocardial infarction (AMI). The median interval from the onset of the AMI to death was 11 days, and from the onset of the AMI to operative closure of the VSD, 14 days. Eight patients died in the operating room or within 2 hours of operation. Death was attributed to unsuccessful VSD closure in 5 patients, to inadequate left ventricular cavity after resection of necrotic myocardium in 5 patients, and to inadequate viable left ventricular myocardium in 4 patients. Heart weights were increased in 14 patients (88%).

674



Gross Description:

Certain cardiac morphologic findings are described in 16 necropsy patients having operative closure of an acquired ventricular septal defect (VSD) during acute myocardial infarction (AMI). The median interval from the onset of the AMI to death was 11 days, and from the onset of the AMI to operative closure of the VSD, 14 days. Eight patients died in the operating room or within 2 hours of operation. Death was attributed to unsuccessful VSD closure in 5 patients, to inadequate left ventricular cavity after resection of necrotic myocardium in 5 patients, and to inadequate viable left ventricular myocardium in 4 patients. Heart weights were increased in 14 patients (88%).

Of the 16 patients, 6 were women (mean age  $69 \pm 7$  years) and 10 were men (mean age  $60 \pm 11$  years). The AMI associated with the VSD was the first coronary event in 13 patients (81%). At least 6 patients had a history of systemic hypertension. Conduction disturbances were diagnosed by electrocardiogram in 5 patients (31%). Coronary artery bypass grafting was performed simultaneously with the VSD closure in 7 patients. The AMI associated with the VSD was anterior in 9 patients and posterior (inferior) in 7. Healed myocardial infarcts were present in 3 patients. All 16 patients had severe ( $>75\%$  in cross-sectional area) narrowing of 1 or more of the 4 major epicardial coronary arteries.

Publication:

Mann JM, Roberts WC. Cardiac morphologic observations after operative closure of acquired ventricular septal defect during acute myocardial infarction: Analysis of 16 necropsy patients. Am J Cardiol (In Press).





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03903-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanisms of severe mitral regurgitation in mitral valve prolapse

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others: C.L. McIntosh, MD  
R.B. Wallace, MD

## COOPERATING UNITS (if any)

Georgetown University Medical Center, Washington, DC

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Certain clinical and mitral valvular morphologic findings are described in 83 patients (aged 26-79 years [mean 60]; 26 women [31%] and 57 men [69%]) with mitral valve prolapse (MVR) and mitral regurgitation (MR) severe enough to warrant mitral valve replacement. In each valve a portion of posterior mitral leaflet was elongated such that the distance from distal margin to basal attachment of this leaflet was similar to the distance from distal margin of anterior leaflet to its basal attachment to left atrial wall. Two major mechanisms for the severe MR were found: 1) dilatation of the mitral annulus with or without rupture of chordae tendineae, and 2) rupture of chordae tendineae with or without dilatation of the mitral annulus.



Gross Description:

Certain clinical and mitral valvular morphologic findings are described in 83 patients (aged 26-79 years [mean 60]; 26 women [31%] and 57 men [69%]) with mitral valve prolapse (MVR) and mitral regurgitation (MR) severe enough to warrant mitral valve replacement. In each valve a portion of posterior mitral leaflet was elongated such that the distance from distal margin to basal attachment of this leaflet was similar to the distance from distal margin of anterior leaflet to its basal attachment to left atrial wall. Two major mechanisms for the severe MR were found: 1) dilatation of the mitral annulus with or without rupture of chordae tendineae, and 2) rupture of chordae tendineae with or without dilatation of the mitral annulus.

All 83 operatively excised valves were examined by the same person and all excised valves had been purely regurgitant (no element of stenosis). No patients had hemodynamic evidence of dysfunction of the aortic valve. Of the 83 patients, 48 (58%) had both dilated annuli (>11 cm in circumference) and ruptured chordae tendineae; 16 (19%) had dilated annuli without ruptured chordae, and 16 (19%) had ruptured chordae without significant annular dilatation. In 3 patients neither the annulus was dilated nor were chordae ruptured, and therefore, the mechanism of the MR in them is uncertain. Mitral chordal rupture was nearly as frequent in the 64 patients with clearly dilated annular circumferences as in the 19 patients with normal or insignificantly dilated annular circumferences ( $\leq 11$  cm).

Publication:

Roberts WC, McIntosh CL, Wallace RB. Mechanisms of severe mitral regurgitation in mitral valve prolapse determined from analysis of operatively excised valves. Am Heart J 1987; 113:1316-1323.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03904-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Idiopathic dilated cardiomyopathy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others: R.J. Siegel, MD  
B.M. McManus, MD

## COOPERATING UNITS (if any)

Cedars-Sinai Medical Center, Los Angeles, CA 90048 (Dept. of Cardiology)

University of Nebraska Medical Center, Omaha, NE 68105 (Dept. of Pathology)

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Certain clinical and cardiac necropsy findings are described in 152 patients aged 16 to 78 years (mean 45) with idiopathic dilated cardiomyopathy (IDC): 109 (72%) were males and 43 (28%) were females. Compared to the women, the men had a significantly ( $p < .05$ ) shorter mean duration of chronic congestive heart failure (CHF) (43 months - vs - 69 months), a higher percentage of habitual alcoholism (40% - vs - 24%), and a higher mean heart weight (632 g - vs - 551 g). Of the 152 patients, 148 (97%) had clinical evidence of chronic CHF.



Gross Description:

Certain clinical and cardiac necropsy findings are described in 152 patients aged 16 to 78 years (mean 45) with idiopathic dilated cardiomyopathy (IDC): 109 (72%) were males and 43 (28%) were females. Compared to the women, the men had a significantly ( $p < .05$ ) shorter mean duration of chronic congestive heart failure (CHF) (43 months - vs - 69 months), a higher percentage of habitual alcoholism (40% - vs - 24%), and a higher mean heart weight (632 g - vs - 551 g). Of the 152 patients, 148 (97%) had clinical evidence of chronic CHF.

The male-to-female ratio among the 58 known alcoholics was 7.3:1 and among the 70 known non-alcoholics, 1.5:1 ( $p < .05$ ). The duration of clinical evidence of CHF was similar among the known alcoholics and the known non-alcoholics (each 50 months). The interval from onset of chronic CHF to death (known in 120 patients) ranged from 1 to 264 months (mean 54). Comparison of the 27 patients surviving  $>72$  months after onset of chronic CHF to the 64 patients surviving  $\leq 36$  months disclosed a significantly higher frequency in the longer survival group of older patients, of women, of habitual alcoholics, of patients with chest pain syndromes, diabetes mellitus, pulmonar emboli, of patients treated with warfarin, and of patients with bigger hearts at necropsy. Each of the 4 patients without chronic CHF died suddenly and sudden death was the initial manifestation of IDC in them. An additional 33 patients also died suddenly but each of them previously had had chronic CHF. Of the 79 patients (of 131 where information available) with either pulmonary or systemic emboli or both, 67 (85%) had either right- or left-sided thrombi or mural endocardial plaques or both, whereas of the 52 patients without emboli, 36 (69%) had intracardiac thrombi or plaques ( $p < .05$ ). Electrocardiograms in the last 6 months of life in 101 patients disclosed atrial fibrillation in 25; complete left (41 patients) or right (6 patients) or indeterminate (4 patients) bundle branch block in 51 patients; QRS voltage indicative of ventricular hypertrophy in 44 patients (left in 39 patients). The patients with compared to those without left bundle branch block had a significantly longer duration of CHF, (83 months - vs - 43 months), larger mean heart weights (628 g - vs - 590 g), and a higher frequency of grossly visible scars (44% - vs - 13%). The total 12-lead QRS amplitude in 35 men ranged from 74 to 250 mm (mean 147) (10 mm = 1 mV) and in the 14 women, from 75 to 243 mm (mean 167). The hearts at necropsy in the women ranged from 360 to 860 g (mean 551) and in the men, 400 to 940 g (mean 632) ( $p < .05$ ). Grossly visible left ventricular scars were observed in 22 (14%) patients. Histologic examination (96 patients) of sections of the left ventricular walls disclosed interstitial or replacement fibrosis in 55 patients (57%), and inflammatory-cell infiltrates in only 5 patients.

Publication:

Roberts WC, Siegel RJ, McManus BM. Idiopathic dilated cardiomyopathy: An analysis of 152 necropsy patients. Am J Cardiol (In Press).





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 03905-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Frequency and significance of mitral anular calcium

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others: H.E. Motamed, MD

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To determine the frequency and significance of mitral anular calcium (MAC) in hypertrophic cardiomyopathy (HC), 43 clinical and morphologic variables in 194 necropsy cases of HC were examined. Of 96 patients  $\leq 40$  years of age, none had MAC. Of the 98 necropsy patients  $> 40$  years of age, 30 (31%) had MAC, and of them 21 (70%) were women. The mean age of the 30 MAC patients was older than that of the 68 non-MAC patients  $> 40$  years of age (66 years-vs-54 years). The frequency of MAC increased with age.



Gross Description:

To determine the frequency and significance of mitral anular calcium (MAC) in hypertrophic cardiomyopathy (HC), 43 clinical and morphologic variables in 194 necropsy cases of HC were examined. Of 96 patients  $\leq 40$  years of age, none had MAC. Of the 98 necropsy patients  $>40$  years of age, 30 (31%) had MAC, and of them 21 (70%) were women. The mean age of the 30 MAC patients was older than that of the 68 non-MAC patients  $>40$  years of age (66 years-vs-54 years). The frequency of MAC increased with age.

MAC was present in 3 (10%) of the 29 patients aged 41 to 50 years; in 6 (18%) of 34 patients aged 51 to 60 years, and in 11 (52%) of 21 patients aged 61 to 70 years, and in 10 (71%) of 14 patients aged 71 to 90 years. Compared to the 68 patients  $>40$  years of age without MAC, the 30 patients  $>40$  years of age with MAC had higher average peak left ventricular systolic pressures (196 mm Hg - vs - 176 mm Hg); a higher percent of the MAC patients had peak left ventricular systolic pressures  $>140$  mm Hg (85% - vs - 50%); a higher percent of the MAC patients had left ventricular to systemic peak systolic pressure gradients at rest  $>20$  mm Hg (85% - vs - 60%) and a lower percent of the MAC patients had no peak systolic pressure gradient between left ventricle and systemic artery at rest (15% - vs - 52%); a higher percent of the MAC patients had calcific deposits in the epicardial coronary arteries (93% - vs - 43%) and in the aortic valve cusps (33% - vs - 6%), and a higher percent of the MAC patients had severe narrowing by atherosclerotic plaques of 1 or more of the 4 major epicardial coronary arteries (47% - vs - 25%). The frequency of a history of systemic hypertension, diabetes mellitus and total serum cholesterol levels  $>200$  mg/dl in the patients with and without MAC was similar. In no patient with MAC by hemodynamic studies was there evidence of obstruction to left ventricular inflow. The frequency of mitral regurgitation by left ventricular angiography was similar in the patients with and without MAC. Whether or not MAC was responsible for the higher average peak left ventricular pressures and the higher frequency of peak systolic pressure gradients between left ventricle and systemic artery in the patients with MAC compared to those without MAC is uncertain.

Publication:

Motamed HE, Roberts WC. Frequency and significance of mitral anular calcium in hypertrophic cardiomyopathy: Analysis of 194 necropsy patients. Am J Cardiol (In Press).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03906-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Prevalence of myocarditis in acquired immunodeficiency syndrome

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

Others: D.W. Anderson, MD, R. Virmani, MD, J.M. O'Leary, MD  
R.E. Cunnion, MD, M. Robinowitz, MD, A.M. Macher, MD  
S.P. Villaflor, MD, J.E. Parrillo, MD

## COOPERATING UNITS (if any)

Walter Reed Army Medical Center, Washington, DC (Dept. of Pathology)  
Armed Forces Institute of Pathology, Washington, DC  
DC General Hospital and Washington Hospital Center, Washington, DC

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

9.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The prevalence of myocarditis and dilated cardiomyopathy in 71 consecutive necropsy patients with the Acquired Immunodeficiency Syndrome (AIDS) was evaluated: myocarditis was found in 37 cases (52%) and was mostly mononuclear, mild and patchy in distribution, including seven (10%) cases with features consistent with dilated cardiomyopathy.



Gross Description:

The prevalence of myocarditis and dilated cardiomyopathy in 71 consecutive necropsy patients with the Acquired Immunodeficiency Syndrome (AIDS) was evaluated: myocarditis was found in 37 cases (52%) and was mostly mononuclear, mild and patchy in distribution, including seven (10%) cases with features consistent with dilated cardiomyopathy.

Dilated cardiomyopathy at necropsy was associated with major cardiac dysfunction, pericardial effusion and increased heart weight ( $379 \pm 42$  [mean  $\pm$  SD] g vs  $320 \pm 86$  g,  $p < 0.05$ ) as compared to the other 64 AIDS patients. Although viral, protozoan, bacterial, fungal and mycobacterial opportunistic pathogens were present in myocardial sections of 7 of 37 myocarditis cases these included only 2 cases of dilated cardiomyopathy. Therefore, the etiology of myocarditis in a majority of AIDS patients remains idiopathic. The cause of death in 45 cases (62%) was pulmonary failure and cardiovascular death in 6 cases (8%) of which 4 had dilated cardiomyopathy. Thus, myocarditis is a frequent necropsy findings in patients with AIDS and it may cause dilated cardiomyopathy and cardiac failure.

Publication:

Anderson DW, Virmani R, Reilly JM, O'Leary TO, Cunnion RE, Robinowitz M, Macher AM, Punja U, Villaflor SP, Parrillo JE, Roberts WC. Prevalence of myocarditis in acquired immunodeficiency syndrome. Journal of the American College of Cardiology (In Press).





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03907-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Frequency of systemic hypertension in various cardiovascular diseases

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: William C. Roberts, MD

## COOPERATING UNITS (if any)

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Cardiac Pathology

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This report summarizes the clinical frequencies of sytemic hypertension and necropsy evidence of cardiomegaly in various cardiovascular conditions. Systemic hypertension is present in >50% of patients with various coronary events, in >75% of patients with various cerebrovascular events, and in >90% of patients with aortic dissection. Hypertension is the sole underlying factor in most patients with nontraumatic cerebral arterial or aortic (dissection = partial rupture) rupture. In association with hypercholesterolemia (serum total cholesterolemia >150 mg/dl), hypertension clearly accelerates atherosclerosis and its devastating consequences.

Publication:

Roberts WC. Frequency of systemic hypertension in various cardiovascular diseases. Am J Cardiol 1987; (In Press).

684



Gross Description:

This report summarizes the clinical frequencies of sytemic hypertension and necropsy evidence of cardiomegaly in various cardiovascular conditions. Systemic hypertension is present in >50% of patients with various coronary events, in >75% of patients with various cerebrovascular events, and in >90% of patients with aortic dissection. Hypertension is the sole underlying factor in most patients with nontraumatic cerebral arterial or aortic (dissection = partial rupture) rupture. In association with hypercholesterolemia (serum total cholesterolemia >150 mg/dl), hypertension clearly accelerates atherosclerosis and its devastating consequences.

Publication:

Roberts WC. Frequency of systemic hypertension in various cardiovascular diseases. Am J Cardiol 1987; (In Press).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03908-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Interobserver variability in the interpretation of endomyocardial biopsies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans, Chief, Ultrastructure Section, PA, NHLBI

Others: J.G. Shanes, J. Ghali, C.C. Tsai, S. Furner and R. Subramanian, University of Illinois, Chicago, Illinois.

M.E. Billingham, Stanford University, Palo Alto, California, J.J. Fenoglio, Columbia University, New York, New York, W.D. Edwards, Mayo Clinic, Rochester, Minnesota, J.E. Saffitz, Washington University, St. Louis, J. Isner, New England Medical Center.

## COOPERATING UNITS (if any)

University of Illinois, Chicago, Illinois, Stanford University, Palo Alto, Calif., Columbia University, New York, NY, Washington University, St. Louis, and New England Medical Center.

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Detailed comparisons were made of the individual interpretations by 7 cardiovascular pathologists, of histologic sections of endomyocardial biopsy specimens from 16 patients with the clinical diagnosis of dilated cardiomyopathy. Analysis of the data showed that interobserver variability is high in interpreting biopsy specimens from patients with dilated cardiomyopathy and that quantitative and standardized methods are needed to improve diagnostic consistency.



Project Description

Objectives: To evaluate interobserver variability in the interpretation of morphologic changes in endomyocardial biopsy specimens from patients with the clinical diagnosis of dilated cardiomyopathy.

Methods used and major findings: Controversy exists over the role of endomyocardial biopsy in evaluating patients with dilated cardiomyopathy, particularly in regard to detecting myocarditis and in assessing prognosis. Interobserver variability, if high, could explain conflicting reports. To assess for this, biopsy specimens from 16 patients with the clinical diagnosis of dilated cardiomyopathy were submitted to 7 cardiac pathologists. The same slides were independently reviewed by each, and assessed for fibrosis, hypertrophy, nuclear changes, mean lymphocyte count per high power field, and presence or absence of myocarditis. The prevalence of significant fibrosis ranged from 25% to 69%; of hypertrophy, from 19% to 88%; of nuclear changes, from 31% to 94%, and of abnormal lymphocyte count, from 0 to 38%. One or more pathologists diagnosed definite or possible myocarditis in 11 of the 16 patients. It was concluded from the study that interobserver variability is high in interpreting biopsy specimens from patients with dilated cardiomyopathy and that quantitative and standardized methods are needed to increase diagnostic consistency.

Significance: The study demonstrates a high degree of interobserver variability in the assessment of morphologic changes in endomyocardial biopsy specimens and underscores the need for standardization of criteria for the evaluation of such changes.

Project course: Project completed.

Publications: Shanes, J. G., Ghali J., Billingham, M. E., Ferrans, V. J., Fenoglio, J. J., Edwards, W. D., Tsai, C. C., Saffitz, J. E., Isner, J., Furner, S., Subramanian, R.: Interobserver variability in the pathologic interpretation of endomyocardial biopsy results. Circulation 1987; 75: 401-405.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03909-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Benzyl alcohol as a storage solution for explanted bioprosthetic heart valves.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans Chief, Ultrastructure Section, PA, NHLBI

Others: S. L. Hilbert Center for Devices and Radiological Health, FDA,  
Rockville, Maryland  
A. Yoganathan Georgia Institute of Technology, Atlanta, Georgia  
M. Jones Senior Surgeon, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Center for Devices and Radiological Health, FDA, Rockville, Maryland  
Georgia Institute of Technology, Atlanta, Georgia  
Surgery Branch, NHLBI

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In vitro performance tests and morphologic studies were conducted to assess the suitability of 1% benzyl alcohol in Dulbecco's buffered saline as a storage solution in which explanted bioprosthetic heart valves can be kept before conducting in vitro testing and morphologic evaluation. The results obtained show that this solution allows for adequate preservation of both mechanical and morphologic properties of bioprosthetic valves.



## Project Description

Objectives: As a result of interest in determining the various failure modes of explanted bioprosthetic heart valves, it became necessary to investigate the feasibility of conducting in vitro functional tests and morphologic studies on the same explanted valves. The major obstacle to these dual studies is the lack of a storage solution that would preserve explanted valve morphology without biasing the results of in vitro testing. Currently, a choice must be made between conducting functional tests or histologic studies on explanted valves, since all routinely used fixatives contain aldehydes which will cross-link proteins and may change the mechanical properties of the leaflets. Therefore, the purpose of this study was to evaluate 1% benzyl alcohol in Dulbecco's phosphate buffered saline as an explanted valve storage solution suitable for both in vitro performance testing and morphologic studies.

Methods used and major findings: An initial study was undertaken to confirm that the presence of aldehydes in a storage solution does alter the in vitro performance of explanted bioprostheses. Unimplanted bioprostheses were rinsed in buffered saline and submerged in ACD-anticoagulated human whole blood at 37 C and gently rocked for 14 days. The valves were rinsed with buffered saline and placed in the following storage solutions for 7 days: buffered saline (pH 7.0), buffered saline - 0.4% glutaraldehyde (pH 7.0), buffered saline - 1% benzyl alcohol (pH 7.1) or buffered saline - 4% formaldehyde - 1% glutaraldehyde (pH 7.1). In vitro performance testing studies were then conducted. Following the completion of the in vitro testing, the valves were placed in 4% formaldehyde - 1% glutaraldehyde in buffered saline for 14 days. Histologic and ultrastructural studies were then initiated. To verify the in vitro results, an in vivo study was conducted in which 10 porcine aortic valve and 2 bovine pericardial valve bioprostheses (25 mm) were implanted in the mitral position in juvenile sheep for a mean of 13 weeks. The explanted valves were stored in buffered saline - 1% benzyl alcohol for 14 days prior to in vitro performance testing. On completion of these studies, the explanted valves were placed in fixative (4% formaldehyde - 1% glutaraldehyde in buffered saline). The performance testing results indicated that the valves stored in 4% formaldehyde - 1% glutaraldehyde buffered saline following exposure to whole blood in vitro show stiffening of the leaflets, as reflected by an increase in the slope of the mean pressure drop (diastole) versus root-mean-square flow rate curve. The in vitro results indicate that performance is not altered by the presence of 1% benzyl alcohol in the storage solution. Morphologic studies demonstrated that the morphology of valvular collagen, elastin and host cells are reasonably well preserved in explanted bioprostheses stored in buffered saline - 1% benzyl alcohol.

Significance: Benzyl alcohol in buffered saline is suitable as a storage solution in which explanted bioprosthetic valves can be kept for conducting morphologic studies and in vitro performance tests.

Project course: Project completed.

Publications: Hilbert, S. L., Ferrans, V. J., Yoganathan, A., Jones, M.: The use of buffered saline - benzyl alcohol as a storage solution for explanted bioprosthetic heart valves. Trans. Soc. Biomater. 1987; 202.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03910-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Lesions produced by dietary deficiency and excess of selenium.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans, Chief, Ultrastructure Section, PA, NHLBI

Others: J. F. Van Vleet, Purdue University, West Lafayette, Indiana

## COOPERATING UNITS (if any)

Purdue University School of Veterinary Medicine, West Lafayette, Indiana

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure

## INSTITUTE AND LOCATION

NHLBI, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A review is presented of the pathologic anatomy of lesions caused by dietary deficiency and excess of selenium in humans and animals. The most important lesions produced by selenium deficiency are necrosis of cardiac and skeletal muscle, but many other organ systems are also affected. Excess of selenium produces peripehral neuropathy and other neurological changes as well as cutaneous lesions.



Project Description

Objectives: To define the pathologic anatomy of lesions produced by dietary deficiency and excess of selenium, with emphasis on changes in the cardiovascular system.

Methods used and major findings: Cardiac and skeletal muscle necrosis are major findings in naturally occurring and experimentally induced deficiency of selenium. However, other organs frequently are sites of lesions of selenium deficiency. These include: liver necrosis (hepatosis dietetica); gastric ulceration involving the region of the esophageal entrance; steatitis; edema, hemorrhage and vascular thrombosis in the skin; alopecia; encephalomalacia; localized axonal dystrophy; testicular degeneration; embryonic death with subsequent resorption; anemia; cataracts; alveolar hemorrhage; necrosis of renal proximal tubular epithelium; pancreatic necrosis, and atrophy of dental enamel. Excessive intake of selenium in animals usually occurs because of ingestion of plants that contain large amounts of selenium (seleniferous plants); in humans, it is usually related to a high content of selenium in water, soil and vegetables, or to high intake of selenium tablest sold as food supplements. Manifestations of selenosis include dry and brittle hair, dermatitis, and peripheral neuropathy and various other neurological changes.

Significance: The lesions of selenium deficiency and excess constitute important problems of theoretical (free radical effects) and clinical (veterinary practice) importance.

Project course: Project completed.

Publications: Van Vleet, J. F., Ferrans, V. J.: Comparative pathology of selenium and vitamin E deficiency and excess. In: Nutritional Diseases. Research Directions in Comparative Pathobiology, Scarpelli, D. G., Migaki, G., editors, Alan R. Liss, Inc., 1986, 359-396.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03911-01 PA

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Neurological complications of cardiovascular therapy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans Chief, Ultrastructure Section, PA, NHLBI

Others: A. C. Cuetter Uniformed Services University of the Health Sciences, Bethesda, Maryland

COOPERATING UNITS (if any)

Uniformed Services University of the Health Sciences, Bethesda, Maryland

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A description is made of the pathologic anatomy and physiology of the major neurologic complications of cardiovascular medical and surgical therapy. These complications are classified according to their etiology, including side effects and toxicity of drug therapy, cardioversion, open heart surgery, artificial heart valves, carotid endarterectomy, and cardiopulmonary resuscitation.

692



### Project Description

Objectives: To define the clinical and anatomic characteristics of neurological complications of cardiovascular therapy.

Methods used and major findings: Major neurological complications of various modalities of cardiovascular therapy are common. In this presentation, the pathophysiologic and morphologic aspects of such complications are classified as follows according to their cause: 1) drug therapy, including antihypertensive medications, antiarrhythmic drugs (lidocaine, tocainide, quinidine, procainamide, dysopyramide, phenytoin, propafenone, lorcaine, flecainide, amiodarone, bretylium), vasodilators for the relief of angina pectoris, digitalis, streptokinase and anticoagulants; 2) cardioversion; 3) open heart surgery; 4) surgery of the thoracic aorta; 5) percutaneous intravascular catheterization and other cardiac invasive procedures; 6) artificial heart valves; 7) cardiac transplantation; 8) carotid endarterectomy, and 9) cardiopulmonary resuscitation.

Significance: Neurological complications represent clinically important aspects of cardiovascular therapy, and this presentation provides a comprehensive overview of these problems.

Project course: Project completed.

Publications: Cuetter, A. C., Ferrans, V. J.: Neurological complications of cardiovascular therapy. In: Current Problems in Cardiology, O'Rourke, R. A., Crawford, M. H., Eds, Vol. 12, 1987, pp 159-211.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03912-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Optical methods for the nondestructive evaluation of collagen in heart valves.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans Chief, Ultrastructure Section, PA, NHLBI

Others: S. L. Hilbert Center for Devices and Radiological Health, FDA,  
Rockville, MarylandW. M. Swanson Department of Mechanical Engineering, Washington  
University, Saint Louis, Missouri

## COOPERATING UNITS (if any)

Center for Devices and Radiological Health, Rockville, Maryland  
Department of Mechanical Engineering, Washington University,  
Saint Louis, Missouri

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An assessment was made of the suitability of optical methods as means of nondestructively evaluating the morphology of collagen in intact leaflets of bioprosthetic valves. The results demonstrate that transmitted polarized light and incident polarized light optics facilitate the imaging of the inherent birefringence of valvular collagen fibers and serve to determine the patterns of collagen configuration and orientation in unimplanted and explanted porcine aortic valvular and bovine pericardial bioprostheses.

697



Project Description

Objectives: To determine whether or not the natural birefringence of collagen can be used to evaluate, by means of polarized light microscopy, the structural arrangement of collagen in implanted and in explanted bioprosthetic heart valves.

Methods used and major findings: The aim of this study was to assess the suitability of nondestructive morphologic methods as a means of evaluating collagen morphology in intact leaflets of bioprosthetic heart valves. The results of this study demonstrate that transmitted polarized light and incident polarized light optics facilitate the imaging of the inherent birefringence of valvular collagen fibers. Polarized light optics readily document the different patterns of collagen orientation and configuration in porcine aortic valvular and bovine pericardial valvular bioprostheses. Incident polarized light optics also provide information on leaflets surface morphology. Verification that the birefringence observed by polarized light optics represents leaflet collagen was provided by conventional histologic and transmission electron microscopic methods. Quantitative determinations of the spacing of collagen bundle waves (degree of crimping) could be made in intact leaflets, and gave similar values in intact and in sectioned pericardial valve leaflets. Potential applications of polarized light optics in the assessment of bioprosthetic valve collagen are as follows: 1) the selection of the desired orientation of collagen bundles within pericardium intended to be configured into bioprosthetic leaflets; 2) evaluation of the effects of mechanical stresses and leaflet motion on collagen morphology in bioprosthetic valve leaflets; and 3) initial screening of leaflet specimens and selection of the desired collagen orientation for embedding and sectioning of samples for conventional morphologic studies.

Significance: The study demonstrates that transmitted and incident polarized light microscopy are extremely useful methods for the nondestructive evaluation of the degree of crimping and the patterns of organization of collagen in the leaflet of bioprosthetic heart valves, and that these methods provide information that cannot be obtained otherwise.

Project course: Project completed.

Publications: Hilbert, S. L., Ferrans, V. J., Swanson, W. M.: Optical methods for the nondestructive evaluation of collagen morphology in bioprosthetic heart valves. J. Biomed. Mater. Res. 1986; 20: 1411-1421.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03913-01 PA

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Myocardial diseases of animals

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans

Chief, Ultrastructure Section, PA, NHLBI

Others: J. F. Van Vleet

Department of Pathology, School of Veterinary  
Medicine, Purdue University, West Lafayette,  
Indiana

COOPERATING UNITS (if any)

Department of Pathology, School of Veterinary Medicine, Purdue  
University, West Lafayette, Indiana

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.3

PROFESSIONAL:

0.2

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An extensive review has been prepared of the gross anatomic, histologic and ultrastructural features of myocardial diseases occurring in animals. Included among these are diseases with known or suspected heritability, diseases produced by nutritional deficiencies, diseases of unknown etiology, diseases of toxic etiology, diseases associated with physical injuries and shock, diseases associated with endocrine disorders, and myocarditis.



Project Description

Objectives: To provide a comprehensive review of the pathologic anatomy of myocardial diseases occurring in animals.

Methods used and major findings: Based on case material gathered by the authors and on review of the literature, descriptions have been prepared of the cardiac gross anatomic, histologic and ultrastructural changes that characterize the myocardial diseases of animals. Naturally occurring and experimentally produced conditions are both considered. Included among these are diseases with known or suspected heritability, diseases produced by nutritional deficiencies, diseases of unknown etiology, diseases of toxic etiology, diseases associated with physical injuries and shock, diseases associated with endocrine disorders, and myocarditis.

Significance: A comprehensive reference source has been provided for evaluating the pathology of myocardial diseases in animals.

Project course: Project completed.

Publications: Van Vleet, J. F., Ferrans, V. J.: Myocardial diseases of animals. Am. J. Pathol. 1986; 124: 98-178.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03914-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ultrastructural abnormalities in diverse types of cardiomyopathies.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans Chief, Ultrastructure Section, PA, NHLBI

Others: E. R. Rodriguez Visiting Fellow, Ultrastructure Section, PA, NHLBI  
Y. Tomita Visiting Fellow, Surgery Branch, NHLBI  
K. Saito Guest Researcher, Ultrastructure Section, PA, NHLBI

## COOPERATING UNITS (if any)

Surgery Branch, NHLBI

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Based mainly on case material accumulated in the Pathology Branch, NHLBI, descriptions are presented of the gross anatomic, histologic and ultrastructural alterations of the heart in different types of cardiomyopathies, including hypertrophic cardiomyopathy, ventricular dilated cardiomyopathy, restrictive cardiomyopathy, the endomyocardial diseases, and various secondary heart muscle diseases, with emphasis on their differential diagnosis.



Project Description

Objectives: To describe the cardiac gross anatomic, histologic and ultrastructural abnormalities of the heart in diverse types of cardiomyopathies.

Methods used and major findings: Based on literature review and the personal experience of the authors, descriptions are presented of the pathologic anatomy of different types of cardiomyopathies. Hypertrophic cardiomyopathy is characterized by concentric, often asymmetric hypertrophy and by severe degrees of myocardial fiber disarray; dilated cardiomyopathy, by dilatation of the ventricular chambers and by a number of degenerative changes in the cardiac myocytes; restrictive cardiomyopathy, either by endocardial thickening or by myocardial fibrosis or infiltrative disorders; secondary cardiomyopathies, by more specific changes, often involving the formation of storage deposits (i.e., iron, glycogen, acid mucopolysaccharides). Emphasis is placed on the differential diagnosis of these disorders.

Significance: An up to date description of the pathologic anatomy of the cardiomyopathies has been provided.

Project course: Project completed.

Publications: Ferrans, V. J., Rodriguez, E. R., Tomita, Y. and Saito, K.: Ultrastructural abnormalities in different types of cardiomyopathies. In: Pathophysiology of Heart Disease, N. S. Dhalla, P. K. Singal and R. E. Beamish, Editors, Martinus Nijhoff Publishing, Boston, 1987, pp 251-268.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03915-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Effect of selenium deficiency on the chronic toxicity of adriamycin in rats

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans Chief, Ultrastructure Section, PA, NHLBI

Others: X. Chen USDA, Beltsville, Maryland  
A. Xue USDA, Beltsville, Maryland  
V. C. Morris USDA, Beltsville, Maryland  
E. H. Herman Division of Drug Biology, FDA, Washington, D.C.  
A. El-Hage Division of Drug Biology, FDA, Washington, D.C.  
O. A. Levander USDA, Beltsville, Maryland

## COOPERATING UNITS (if any)

USDA-ARS, Beltsville Human Nutrition Research Center, Vitamin and  
Mineral Nutrition Laboratory, Beltsville, Maryland 20705  
Division of Drug Biology, FDA, Washington, D.C.

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.1

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A study was made of the effect of selenium deficiency on the chronic toxicity produced by doxorubicin in rats fed diets adequate in vitamin E. The severity of the cardiotoxicity was unaltered by selenium deficiency; however, the growth-inhibiting effect and the nephrotoxicity produced by doxorubicin were more marked in the selenium-deficient animals.



Project Description

Objectives: To assess the effect of selenium deficiency on the chronic toxicity of adriamycin in rats.

Methods used and major findings: Considerable evidence supports the concepts that: 1) selenium and vitamin E, which function as components of the glutathione peroxidase system, play a major role in the free radical scavenging that mediates the defense mechanisms of the heart against peroxidative damage, and 2) that adriamycin causes cardiotoxicity through mechanisms that involve the formation of oxygen free radicals. Therefore, it seemed of interest to examine the effect of selenium deficiency on the chronic toxicity of adriamycin in rats fed diets adequate in vitamin E. Selenium-deficient and -supplemented diets were fed to rats for 10 weeks, after which groups of 10 rats fed each diet were given weekly intravenous injections of adriamycin in saline at doses of 0, 0.5, or 1.0 mg per kg body weight for 12 weeks. All rats were killed at 24 weeks, and histologic and electron microscopic studies were made to assess the severity of the cardiomyopathy and the nephropathy produced by adriamycin. Even though the cardiac glutathione peroxidase activity in the selenium-deficient group was less than one percent than that in the selenium-supplemented group, the severity of the adriamycin-induced cardiomyopathy was similar in both groups. However, the selenium-deficient rats were more sensitive to the growth-inhibiting effect of the higher dose of adriamycin than the selenium-supplemented rats. Moreover, the lower dose of adriamycin caused a mild nephropathy in 70% of the deficient rats, but affected only 10% of the supplemented rats.

Significance: It was surprising to find that the cardiac lesions produced by adriamycin were not worsened by selenium deficiency. However, these findings are in accord with observations showing that iron is involved in the doxorubicin-mediated formation of free radicals, and that other free radical scavengers are only of very limited usefulness in blocking the damage produced by these free radicals.

Project course: Project completed.

Publications: Chen, X., Xue, A., Morris, V. C., Ferrans, V. J., Herman, E. H., El-Hage, A., Levander, O. A.: Effect of selenium deficiency on the chronic toxicity of adriamycin in rats. J. Nutr. 1986; 116: 2453-2465.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03916-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of protection by ICRF-187 against alloxan-induced diabetes in mice.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans Chief, Ultrastructure Section, PA, NHLBI

Others: A. El-Hage Division of Drug Biology, FDA, Washington, DC  
E. H. Herman Division of Drug Biology, FDA, Washington, DC  
G. C. Wang Division of Drug Biology, FDA, Washington, DC  
R. K. Crouch Dept. of Ophthalmology, Medical University of  
South Carolina, Charleston, SC

## COOPERATING UNITS (if any)

Division of Drug Biology, FDA, Washington, DC  
Dept. of Ophthalmology, Medical University of South Carolina

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Pharmacologic studies were made to evaluate the mechanism by which ICRF-187 protects against alloxan-induced diabetes mellitus in mice. These studies led to the conclusion that the most likely mechanism for this protection involves chelation by ICRF-187 of iron ions necessary for the in vivo formation of the reactive oxygen radicals that mediate alloxan-induced damage to the pancreatic beta cells.

702



Project Description

Objectives: To investigate the mechanism by which ICRF-187 protects against alloxan-induced diabetes in mice.

Methods used and major findings: ICRF-187, (+)-1,2-bis(3,5-dioxopiperazin-1-yl)propane, has been shown to protect against alloxan diabetes as well as against doxorubicin-induced cardiotoxicity. Since alloxan-induced pancreatic beta cell damage is thought to be mediated through the generation of highly reactive oxygen radicals by a metal-catalyzed reaction involving both superoxide anion and hydrogen peroxide, in the present study the protective activity of ICRF-187 was compared with that of free radical scavengers, microsomal enzyme inhibitors and chelating agents. The free radical scavengers DMSO, vitamin E and WR2721 markedly reduced alloxan-induced hyperglycemia. ICRF-187 was found not to interact with superoxide anions, and there is no evidence to indicate that any of the known biological effects of ICRF-187 are mediated through free radical scavenging activity. SKF-525 and cimetidine, known inhibitors of drug metabolizing enzymes, also protected against the diabetogenic action of alloxan. Since it was found that ICRF-187 did not alter hexobarbital sleeping time, this compound must protect by mechanism other than microsomal enzyme inhibition. Since the chelating agents EDTA and DETAPAC also were found to protect against alloxan diabetes, ICRF-187 or its hydrolytic products, which are structurally similar to EDTA, could function as chelating agents. Transitional metals such as iron, zinc and copper were found to bind preferentially to a hydrolysis product of ICRF-187.

Significance: The results of this study provide additional support for the concept that chelation of iron by ICRF-187 or its hydrolytic products decreases in vivo formation of reaction oxygen radicals and provides a means for protecting against chronic anthracycline cardiotoxicity and alloxan diabetes.

Project course: Project completed.

Publications: El-Hage, A., Herman, E. H., Yang, G. C., Crouch, R. K., Ferrans, V. J.: Mechanism of the protective effect of ICRF-187 against alloxan-induced diabetes in mice. Res. Commun. Chem. Pathol. Pharmacol. 1986; 52: 341-360.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03917-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cardiovascular diseases of swine

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans Chief, Ultrastructure Section, PA, NHLBI

Others: J. F. Van Vleet Department of Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

## COOPERATING UNITS (if any)

Department of Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

0.2

## PROFESSIONAL:

0.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A survey was made of naturally occurring and experimentally induced cardiovascular diseases in swine. Spontaneous diseases include congenital anomalies, endocardial, pericardial, myocardial and vascular lesions, lesions induced by toxins, and cardiac tumors. Experimentally induced lesions include cardiac hypertrophy and lesions induced by chemical agents, acceleration stress, hemorrhagic shock, myocardial ischemia and nutritional deficiencies.



Project Description

Objectives: To survey the pathologic anatomy of spontaneously occurring and experimentally induced cardiovascular diseases in swine.

Methods used and major findings: Descriptions are presented, based on review of the literature and the experience of the authors, of the pathologic anatomy of spontaneously occurring and experimentally induced cardiovascular diseases in swine. Spontaneously occurring diseases considered include: congenital anomalies, pericarditis, endocarditis, other endocardial lesions, hypertrophic and congestive cardiomyopathies, myocarditis, myocardial necrosis in porcine stress syndrome, malignant hyperthermia and herztod, myocardial alterations induced by chemical toxins, rhabdomyomatosis, atherosclerosis, cerebrospinal angiopathy and various types of arteritis. Experimentally induced disorders include: lesions induced by chemical toxins (cobalt, doxorubicin, minoxidil, isoproterenol and monensin), ventricular hypertrophy, acceleration stress, hemorrhagic shock, myocardial ischemia and nutritional deficiencies.

Significance: A comprehensive, up to date review of the pathology of cardiovascular diseases in swine has been provided.

Project course: Completed.

Publications: Van Vleet, J. F., Ferrans, V. J.: Cardiovascular diseases of swine. In: Swine in Cardiovascular Research, Stanton, H. C., and Mersmann, H. J., editors, CRC Press, Boca Raton, Florida, Vol. I, 1986, pp 121-167.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03918-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Inherited cardiac calcinosis in DBA/2 mice

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans Chief, Ultrastructure Section, PA, NHLBI

Others: J. F. Van Vleet Department of Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

## COOPERATING UNITS (if any)

Department of Pathology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana 47907

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.1

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Histologic and electron microscopic studies were made of the pathogenesis of cardiac dystrophic calcinosis, an inheritable condition, in DBA/2 mice. The initial event was mineralization of subepicardial myocytes. This change appeared to first involve the mitochondria, progressing to necrosis and calcification of entire myocytes.



## Project Description

Objectives: To define the morphologic features of dystrophic cardiac calcinosis in DAB/2 mice.

Methods used and major findings: Cardiac dystrophic calcinosis, an inherited condition in DBA/2 mice, produces extensive calcific lesions in right ventricular myocardium. Male weanling mice (n=135) were fed a commercial diet, or a basal semipurified selenium-vitamin E deficient diet with or without added Ag for 15, 20 or 25 weeks. The severity of the cardiac calcinosis was increased in mice which were fed the basal diet with Ag and became debilitated, but was not altered by differences in age or dietary fat content. The morphogenesis of the cardiac alterations was evaluated by microscopic and ultrastructural study of the right ventricular lesions. The initial event was necrosis and mineralization of subepicardial myocytes. Mineral deposits were seen as dense granular and spicular deposits involving either mitochondria only, mitochondria and adjacent sarcoplasm, or the entire sarcoplasm in necrotic myocytes. In mature lesions, the remnants of necrotic myocytes were seen as scattered dense masses of mineralized debris with surrounding fibroplasia and occasional macrophages and giant cells.

Significance: The study shows that dystrophic myocardial calcification in DAB/2 mice develops in association with myocardial necrosis and initial deposition of calcific deposits in mitochondria. The peculiar gross anatomic distribution of these lesions remains unexplained.

Project course: Project completed.

Publications: Van Vleet, J. F., Ferrans, V. J.: Inherited cardiac calcinosis in DBA/2 mice: Ultrastructural changes and dietary effects. Am. J. Vet. Res. 1987; 48: 255-261.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03919-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evaluation of acute and chronic cardiotoxicity in miniature swine.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans Chief, Ultrastructure Section, PA, NHLBI

Others: E. H. Herman Division of Drug Biology, FDA, Washington, DC  
R. S. K. Young Division of Drug Biology, FDA, Washington, DC

## COOPERATING UNITS (if any)

Division of Drug Biology, FDA, Washington, DC

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An evaluation was made of the suitability of miniature swine as animal models for the morphologic assessment of changes related to the acute and the chronic cardiotoxicity produced by pharmacologic agents. Minoxidil and doxorubicin were used as experimental agents capable of producing acute and chronic cardiotoxicity, respectively. The results obtained show that miniature swine are useful models to study both types of cardiotoxicity.



Project Description

Objectives: To evaluate the suitability of miniature pigs for the morphologic assessment of acute and chronic cardiotoxicity produced by pharmacologic agents.

Methods used and major findings: Light and electron microscopic studies were undertaken to determine the suitability of miniature swine as animal models for the detection of acute and chronic cardiotoxicity. In the acute study, animals were dosed orally with 1, 3 or 10 mg/kg of minoxidil, a vasodilating antihypertensive agent, on 2 consecutive days. Mean arterial pressure decreased and heart rate increased most consistently after the 10 mg/kg dose. However, all 3 dose levels of minoxidil induced myocardial hemorrhages and/or left ventricular papillary muscle necroses in animals euthanatized 48 hours after the second dosing. The severity of both lesions was dose-dependent. Chronic cardiotoxicity (chronic cardiomyopathy) was induced by doxorubicin, an anthracycline drug with antineoplastic properties. Miniature swine were given 6 IV injections of either 1.6 or 2.4 mg/kg doxorubicin at 3 week intervals. Three weeks after the last dosing, myocardial lesions were present in all animals. The lesions were more severe at the higher dose. The cardiomyopathy was comparable to that found in human patients and consistently displayed two characteristics, cytoplasmic vacuolization and myofibrillar loss.

Significance: These studies demonstrate that miniature swine are useful as large animal models to assess morphologic problems related to acute and chronic cardiac toxicity.

Project course: Project completed.

Publications: Herman, E. H., Young, R. S. K., Balazs, T., Ferrans, V. J.: The evaluation of acute and chronic cardiotoxicity in miniature swine. In: Swine in Biomedical Research, Tumbleson, M. E., editor, Plenum Publishing Corporation, Vol. 3, 1986, 1659-1670.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03920-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Cardiac Toxicity

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans

Chief, Ultrastructure Section, PA, NHLBI

Others: T. Balazs

Division of Drug Biology, FDA, Washington, D.C.

J. Hanig

Division of Drug Biology, FDA, Washington, D.C.

E. H. Herman

Division of Drug Biology, FDA, Washington, D.C.

## COOPERATING UNITS (if any)

Division of Drug Biology, FDA, Washington, D.C.

## LAB/BRANCH

Pathology Branch, NHLBI

## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An extensive description was made of pharmacologic, pathophysiologic and morphologic changes involved in cardiac toxicity produced by drugs and chemical agents.



Project Description

Objectives: To explore the relationships between morphologic changes and biochemical mechanisms involved in various types of cardiac toxicity induced by drugs and chemicals.

Methods used and major findings: The reactions of the heart to toxic injury can be manifested in the form of cardiac hypertrophy, cardiomyopathies, cardiac necrosis, hypersensitivity myocarditis, toxic myocarditis, pericarditis, endocardial mural and valvular fibrosis, toxic vasculitis and hypersensitivity vasculitis. The correlations between morphologic, biochemical and pharmacologic aspects of each of these types of lesions are discussed in detail.

Significance: This review provides an overview of the problems encountered in the differential diagnosis of morphologic changes associated with toxic injury to the heart.

Project course: Project completed.

Publications: Balazs, T., Ferrans, V. J., Hanig, J. and Herman, E. H. : Cardiac toxicity. In: Target Organ Toxicity, 1986; 2: 19-43.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03921-01 PA

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Pathology of bioprosthetic cardiac valves

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. J. Ferrans

Chief, Ultrastructure Section, PA, NHLBI

Others: Y. Tomita

Visiting Fellow, Surgery Branch

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## SECTION

Ultrastructure Section

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Based on the experience of the Pathology and Surgery Branches, NHLBI, a description is presented of the morphology of unimplanted cardiac valvular bioprostheses and of the changes that develop in these devices after implantation as substitute cardiac valves. Emphasis is placed on the problems of valvular tears and calcific deposits, the two most important complications that develop in implanted cardiac valvular bioprostheses.



## Project Description

Objectives: To provide a comprehensive description of the morphologic features of unimplanted bioprosthetic cardiac valves and of the morphologic changes that develop in these devices after being implanted as substitute valves.

Methods used and major findings: A review is presented of the morphology of unimplanted cardiac valvular bioprostheses and of the changes that develop in these devices after implantation as substitute cardiac valves. The changes are evaluated on the basis of radiographic, gross anatomic, histologic, and transmission and scanning electron microscopic observations. Porcine aortic valvular bioprostheses, bovine pericardial bioprostheses, dura mater valves, fascia lata valves and aortic homografts are considered. Changes that are best or, in some instances, only observed with the bioprosthesis in situ include: perivascular leak; obstruction to the left ventricular outflow tract or the coronary ostia; entanglement of sutures; disproportion; angulation, and compression of an anomalous left main or circumflex coronary artery. Changes that involve the bioprosthesis itself include: adherence of platelets and inflammatory cells to the valve surfaces; surface thrombi; lining of the surfaces by a fibrous tissue sheath which can be the cause of cuspal stiffening and stenosis; infection; tears and perforations; calcification; intracuspal hematomas; bending of the struts, and deposits of lipids and amyloid. From the functional standpoint the most important of these changes are calcification, which directly involves collagen, and tears and perforations, which are consequences of collagen breakdown.

Significance: The durability of bioprosthetic valves is limited because they undergo progressive, time-dependent deterioration. An understanding of this process of deterioration is being obtained through morphologic analysis of failing valves and is necessary for designing a new generation of more durable valves.

Project course: Project completed.

Publications: Ferrans, V. J., Tomita, Y., Hilbert, S. L., Jones, M. and Roberts, W. C.: Pathology of bioprosthetic valves. Hum. Pathol. 1987; 18: 586-595.



Annual Report of the Pulmonary Branch  
National Heart, Lung and Blood Institute  
October 1, 1986 through September 30, 1987

The research of the Pulmonary Branch centers on diseases of the alveolar structures, the site in the body at which gases are exchanged between air and blood. Three categories of common diseases are investigated: all represent chronic inflammatory disorders of the lower respiratory tract in which the inflammation causes the changes in the lung parenchyma that defines the clinical presentation of each disease.

(1) Disorders characterized by extensive fibrosis of the lung parenchyma. These disorders represent a large subgroup of the interstitial lung disorders, chronic disorders in which derangements of the alveolar wall are accompanied by "fibrosis," an accumulation of mesenchymal cells and the collagenous matrix produced by these cells. In general, the inflammation of these disorders are dominated by alveolar macrophages, and to a less extent, neutrophils and/or eosinophils. Examples of these fibrotic disorders include idiopathic pulmonary fibrosis and asbestosis.

(2) Disorders characterized by the accumulation of T-lymphocytes in the lower respiratory tract. These disorders are also a subgroup of the interstitial lung disorders. However, although occasionally associated with progressive fibrosis of the lung parenchyma late in their course, these disorders are universally characterized by large numbers of T-lymphocytes that dominate the inflammation and cause dysfunction by their presence which distorts the alveolar, bronchial, and vascular walls, thus modifying the intimate relationship between air and blood. Examples of the T-lymphocytes disorders include sarcoidosis and berylliosis.

(3) Disorders characterized by destruction of the alveolar walls. These disorders are commonly called emphysema. Of the  $2 \times 10^6$  individuals in the USA with emphysema, approximately 98% acquire the disease, usually on the basis of cigarette smoking, while 2% have an inherited disorder called alpha 1-antitrypsin deficiency. All forms of emphysema are characterized by a dissolution of the lung parenchyma. The inflammation that causes these changes is dominated by alveolar macrophages together with smaller numbers of neutrophils.

The inflammation of all of these disorders can be evaluated by bronchoalveolar lavage, a technique that permits direct access to the epithelial surface of the lower respiratory tract where inflammatory cells and relevant molecules in the epithelial lining fluid can be easily and repetitively sampled. To accomplish bronchoalveolar lavage, local anesthesia is administered to the upper respiratory tract and a fiberoptic bronchoscope is gently wedged into a distal bronchus. Sterile saline is infused into the bronchoscope and then suctioned back, thus recovering the epithelial lining fluid of the lower respiratory tract. Over the past decade, this procedure has been used by the Pulmonary Branch to evaluate several thousand individuals.

I. Disorders Characterized by Extensive Fibrosis of the Lung Parenchyma



The current concepts of the mechanisms of pulmonary fibrosis hold that the accumulation of fibroblasts and fibroblast products results from two general processes. First, there must be damage to the alveolar wall; in most cases this is mediated by products of inflammatory cells, including macrophages, neutrophils and eosinophils. Second, there is enhanced proliferation of mesenchymal cells in the alveolar walls driven by growth signals released in the local milieu.

Although mononuclear phagocytes are known to be capable of releasing a variety of growth signals for fibroblasts, recent observations have demonstrated that one of these molecules is platelet-derived growth factor (PDGF) a 31 kDa dimeric glycoprotein that is potent both as a chemoattractant for mesenchymal cells as well as a stimulus for mesenchymal cells to enter the G1 phase of the cell cycle. Blood monocytes do not normally release PDGF, but do so when stimulated. When matured in culture, monocytes spontaneously release PDGF and tissue macrophages recovered from internal organs spontaneously release low levels of this potent mediator. The interest in PDGF as an important mediator of normal and pathologic scar formation has been heightened by the observation that the B-chain of PDGF is coded for by the c-sis proto-oncogene, a 30 kb gene localized to region q11>q21 of chromosome 22. Interestingly, while the v-sis oncogene (the retroviral analog of the human c-sis gene) was originally recovered from a naturally occurring sarcoma of a Woolly monkey and is capable of transforming normal fibroblasts into continuously proliferating cells in vitro, recent studies have shown that activated blood monocytes, in vitro matured monocytes, and alveolar macrophages all express the c-sis proto-oncogene as well as produce and secrete PDGF. Together, these observations support the concept that, in addition to a possible role in malignancy, activation of the c-sis proto-oncogene could play a key role in the pathogenesis of tissue fibrosis. To evaluate this concept, alveolar macrophages were recovered from patients with idiopathic pulmonary fibrosis (IPF), a fibrotic disorder characterized by replacement of the normal alveolar walls by scar tissue dominated by the accumulation of mesenchymal cells. Since normal alveolar macrophages express c-sis proto-oncogene and release low levels of PDGF, and since IPF is likely initiated by immune complexes activating alveolar macrophages in the lower respiratory tract, we hypothesized that normal alveolar macrophages stimulated with immune complexes would be stimulated to release increased amounts of PDGF and alveolar macrophages recovered from individuals with IPF would spontaneously release exaggerated amounts of active PDGF. Evaluation of normal alveolar macrophages stimulated with immune complexes demonstrated several-fold increase in PDGF release. Importantly, evaluation of alveolar macrophages recovered from the lungs of IPF patients demonstrated spontaneous release of 4-fold more PDGF than alveolar macrophages recovered from normal individuals. Furthermore, these PDGF molecules were active, as evidenced by their chemotactic activity for smooth muscle cells and ability to act as a "competence" factor for fibroblast growth. Together, these observations suggest the possibility that mesenchymal cell accumulation within the alveolar walls in this fibrotic disorder may result, in part, from the exaggerated release of PDGF by mononuclear phagocytes in the lower respiratory tract.

Since normal human platelet PDGF molecules are dimers of A-and B-chains and platelets and monocytes are derived from the same marrow precursors, it is





possible that in addition to the PDGF B-chain (the c-sis gene product), mononuclear are also capable of expressing the PDGF A-chain. To evaluate this concept, activated blood monocytes were simultaneously evaluated for the expression of PDGF A-and B-chain genes. Human blood monocytes were purified and cultured with and without activation by lipopolysaccharide and poly(A)+ RNA evaluated using Northern analysis and <sup>32</sup>P-labeled A-chain and B-chain (human c-sis) probes. Unstimulated blood monocytes did not express either A-chain or B-chain genes. In contrast, activated monocytes expressed a 4.2 kb mRNA B-chain transcript at 4 hr, but the B-chain mRNA levels declined significantly over the next 18 hr. In comparison, activated monocytes expressed very little A-chain mRNA at 4 hr, but at 12 hr 1.9, 2.3, and 2.8 kb transcripts were observed and persisted through 24 hr. Thus, activation of blood monocytes is followed by PDGF B-chain gene expression preceding PDGF A-chain gene expression, suggesting a difference in the regulation of the expression of the genes for these two chains by these cells.

Fibronectin is a 440,000 dalton dimeric glycoprotein produced by a variety of cells and capable of functioning in a broad range of biologic processes including cell adhesion, spreading, cytoskeletal organization, migration, proliferation and differentiation. Fibronectin accomplishes these diverse processes by acting as a ligand between cells and macromolecules through the use of structural domains in the fibronectin molecule with affinity for collagen, heparin, fibrin, DNA and cell surfaces. Several studies have demonstrated that alveolar macrophages synthesize and secrete fibronectin and that alveolar macrophages associated with chronic inflammatory diseases of the lower respiratory tract secrete exaggerated amounts of this molecule. In the context of the known functions of fibronectin, it is likely that it represents one of the alveolar macrophage mediators that plays a role in both defending the lung and in modulating the damage and repair that accompanies inflammatory processes in the lower respiratory tract. To gain insight into the processes that modulate the level of fibronectin secretion by these cells, monocytes, in vitro matured monocytes and alveolar macrophages were used as models to compare fibronectin mRNA levels and fibronectin secretion in a variety of circumstances. Using Northern analysis and dot-blot analysis with a <sup>32</sup>P-labeled human fibronectin cDNA probe, steady state mRNA levels were evaluated. Human fibronectin specific enzyme-linked immunoassay was used to evaluate fibronectin secretion. In all cases the amounts of fibronectin secreted paralleled fibronectin mRNA levels. Specifically: (1) when fibronectin mRNA was undetectable, as in the case of normal blood monocytes, no fibronectin was secreted, but whenever fibronectin mRNA was present, as in normal alveolar macrophages, fibronectin was secreted by the cells; (2) as monocytes matured into macrophages in vitro, the cells began to express fibronectin mRNA and the cells secreted fibronectin; (3) when alveolar macrophages were activated with surface stimuli such as lipopolysaccharide (LPS) or immune complexes, fibronectin mRNA levels decreased and in parallel, the cells secreted less fibronectin; (4) in IPF, alveolar macrophages contained several fold more fibronectin mRNA transcripts than normal and the cells spontaneously secreted several fold more fibronectin than normal; and (5) when IPF alveolar macrophages were placed in culture, the fibronectin mRNA levels in the cells decreased with time, and concurrently, the amounts of fibronectin produced per unit time continually decreased. The observation of a strict



concordance of fibronectin mRNA levels and fibronectin release by mononuclear phagocytes, suggests that, at least in many circumstances, fibronectin secretion by mononuclear phagocytes is controlled by steady state levels of fibronectin mRNA.

In the context of the observations that the amount of fibronectin produced by mononuclear phagocytes parallels the average levels of fibronectin mRNA in these cells, a study was designed to evaluate whether the steady state fibronectin mRNA levels observed in a population of alveolar macrophages reflect the fibronectin mRNA levels for all macrophages in the population, or whether fibronectin mRNA levels vary from cell to cell. To approach this question, we utilized in situ hybridization techniques with fibronectin RNA probes to evaluate the proportion of alveolar macrophages expressing fibronectin mRNA transcripts to determine whether there are differences in the morphological characteristics of the macrophages expressing or not expressing the fibronectin gene, and, for those macrophages expressing the fibronectin gene, whether there are differences in the number of fibronectin mRNA copies per cell. As models for circumstances in which in vivo fibronectin expression may differ on a cell-to-cell basis, we utilized alveolar macrophages from normal individuals and from patients with IPF. Utilizing in situ hybridization of  $^{35}\text{S}$ -labeled anti-sense and sense RNA fibronectin probes, the study demonstrated that most, but not all, normal alveolar macrophages contain fibronectin mRNA transcripts, and that among those macrophages expressing this gene, the relative amount of fibronectin mRNA transcripts varies from cell to cell. Interestingly, while  $66 \pm 3\%$  of normal alveolar macrophages contain fibronectin mRNA transcripts, this is increased to  $82 \pm 2\%$  ( $p < 0.01$ ) of alveolar macrophages recovered from the lungs of individuals with IPF, a disorder associated with exaggerated amounts of fibronectin in the lower respiratory tract. Furthermore, of the macrophages expressing the fibronectin gene, those from IPF patients contain more fibronectin mRNA transcripts than those from normals. Consistent with this observation, evaluation of tissue samples from IPF patients demonstrated that of all cells present, alveolar macrophages showed the greatest numbers of fibronectin mRNA transcripts per cell. These observations demonstrate that there can be marked cell-to-cell variation in the expression of the gene for a macrophage product such as fibronectin, suggesting that there are processes that modulate similar cells in the same anatomic compartment to vary their expression of the same gene.

Although alveolar macrophages are thought to play an important role in fibrogenesis by releasing mediators that have the capacity to recruit mesenchymal cells and stimulate them to proliferate, under resting conditions, lung fibroblast renewal proceeds slowly, suggesting that suppressive signals may be operating in the milieu of the lower respiratory tract to prevent uncontrolled mesenchymal cell accumulation. In this regard, we hypothesized that alveolar macrophages may express the gene for transforming growth factor- $\beta$  (TGF- $\beta$ ), a 25 kd dimeric protein that suppresses the growth response of many normal cells. To evaluate this hypothesis, RNA was recovered from alveolar macrophages of normal individuals, and evaluated for the presence of TGF- $\beta$  mRNA using Northern analysis and a  $^{32}\text{P}$ -labeled TGF- $\beta$  probe. Autoradiograms revealed that resting alveolar macrophages contained 2.5 kb TGF- $\beta$  specific mRNA transcripts, i.e., resting alveolar macrophages constitutively express the TGF- $\beta$  gene. Interestingly, when the



alveolar macrophages were stimulated with LPS, a classical "model" stimulant of inflammation, there was no upregulation of the TGF- $\beta$  gene. In contrast to the manner in which the TGF- $\beta$  was modulated, the LPS stimulated macrophages produced more PDGF, a potent mitogen for fibroblast proliferation. Thus, the resting and stimulated cells expressed similar amounts of the TGF- $\beta$  gene, but the stimulated cells produced far more of the positive growth signal PDGF. Consistent with the concept of a suppressive role for TGF- $\beta$ , over an 18-hr period in serum free conditions, purified recombinant TGF- $\beta$  was able to suppress PDGF + insulin driven proliferation of human lung fibroblasts. In this context, by releasing TGF- $\beta$  into the local milieu of the lower respiratory tract, resting alveolar macrophages may play a role in suppression of uncontrolled mesenchymal cell accumulation. However, when activated, the alveolar macrophages armamentarium of positive growth signals is probably sufficient to override the suppressive effect of TGF- $\beta$ .

Among the mediators that mononuclear phagocytes are capable of producing is tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; also called "cachectin"), a multimeric protein consisting of 17 kd subunits that is cytotoxic for tumors, has growth activity for fibroblasts, and has systemic effects such as fever and cachexia. To evaluate the control of TNF- $\alpha$  expression by mononuclear phagocytes, we investigated the ability of normal human blood monocytes and alveolar macrophages to express the TNF- $\alpha$  gene and secrete TNF- $\alpha$ . Evaluation of mononuclear phagocyte mRNA by Northern analysis with a  $^{32}\text{P}$ -labeled TNF- $\alpha$  cDNA probe demonstrated that resting monocytes and alveolar macrophages expressed very little TNF- $\alpha$  mRNA. In contrast, when stimulated with LPS, both blood monocytes and alveolar macrophages demonstrated the 1.8 kb mRNA transcripts specific for TNF- $\alpha$ . In parallel, supernatants from monocytes and alveolar macrophages cultured for 24 hr with LPS had TNF- $\alpha$  activity as demonstrated by cytolysis of mouse L-929 fibroblasts that was abrogated by a specific anti-TNF- $\alpha$  antibody and was markedly suppressed when the LPS-stimulated mononuclear cells were incubated in the presence of cycloheximide. This mononuclear phagocyte cytolytic activity co-eluted on a molecular sieve with recombinant human TNF- $\alpha$ , further identifying it as TNF- $\alpha$ . Interestingly, quantification of the TNF- $\alpha$  activity released by monocytes and alveolar macrophages demonstrated that, on a per cell basis using a standard LPS stimulus, alveolar macrophages released 7-fold more TNF- $\alpha$  than did blood monocytes. Thus, while both monocytes and alveolar macrophages are capable of expressing the TNF- $\alpha$  gene when stimulated, alveolar macrophages appear to be more capable as TNF- $\alpha$  producers. This capacity is in contrast to the relatively poor capacity of the alveolar macrophages to produce interleukin-1, a mediator with a very similar spectrum of activity.

One prominent feature of IPF is the marked changes to the alveolar epithelial cells, including destruction of type I alveolar epithelial cells and repopulation of the epithelial surface by type II alveolar epithelial cells and bronchiolar epithelial cells. Alveolar macrophages and neutrophils dominate the inflammatory cell population in the lower respiratory tract of patients with IPF. Because both cell types are capable of inducing oxidant-mediated lung parenchymal cell cytotoxicity, one mechanism to explain the epithelial cell injury associated with IPF is the spontaneous release of toxic oxidants by alveolar macrophages and neutrophils. In



addition, when activated, neutrophils can also release myeloperoxidase, a 115 kDa protein which can interact with  $H_2O_2$ , a product of both mononuclear phagocytes and neutrophils, to form the highly toxic hypohalide anion. In this regard, it is reasonable to hypothesize that the alveolar epithelial cell injury that characterizes IPF may result, at least in part, from an enhanced oxidant burden that may exist in the lower respiratory tract of these patients. To evaluate this concept, inflammatory cells recovered by bronchoalveolar lavage from the lower respiratory tract of patients with IPF were evaluated for their ability to spontaneously cause oxidant-mediated alveolar epithelial cell cytotoxicity in the presence and absence of epithelial lining fluid (ELF) from the same individuals. The IPF cells were spontaneously releasing exaggerated amounts of superoxide ( $O_2^-$ ) and  $H_2O_2$  compared with normal ( $p < 0.02$ ). Cytotoxicity to the alveolar epithelial (AKD) cells was markedly increased when IPF inflammatory cells were incubated with autologous ELF ( $p < 0.02$ ). The majority of IPF patients had ELF myeloperoxidase levels above normal ( $p < 0.002$ ). Incubation of IPF ELF with AKD cells in the presence of  $H_2O_2$  caused increased cellular injury ( $p < 0.01$  compared with control), which was suppressed by methionine, a myeloperoxidase system scavenger. IPF patients with high concentrations of ELF myeloperoxidase deteriorated more rapidly than those with low ELF myeloperoxidase ( $p < 0.05$ ). Thus, IPF is characterized by an increased spontaneous production of oxidants by lung inflammatory cells, the presence of high concentrations of myeloperoxidase in the ELF of the lower respiratory tract, and a synergistic cytotoxic effect of alveolar inflammatory cells and ELF on lung epithelial cells, suggesting oxidants may play a role in causing the epithelial cell injury of this disorder.

Although lung parenchymal cells have an array of intracellular antioxidants to protect themselves from toxic oxidants, much of the oxidant burden imposed on the lower respiratory tract is brought to the extracellular milieu of the parenchymal cells by activated inflammatory cells, inhaled gases, and xenobiotics. In this context, we hypothesized that the extracellular milieu of the alveolar structures may contain macromolecules with antioxidant properties which serve as a first line of defense against oxidants generated outside of the parenchymal cells. To evaluate this concept, a model of extracellular oxidant mediated lung parenchymal cell injury was developed using  $^{51}Cr$ -labeled human lung fibroblasts (HFL-1) exposed to a  $H_2O_2$  generating system (glucose, glucose oxidase) for 8 hr,  $37^\circ$ . Using this model, alveolar epithelial lining fluid (ELF) obtained from healthy nonsmokers by bronchoalveolar lavage was tested for its ability to protect lung parenchymal cells against injury initiated by  $H_2O_2$ . The ELF provided marked antioxidant protection to the lung cells when incubated with the  $H_2O_2$  generating system. Characterization of the antioxidant properties of the macromolecules in ELF demonstrated its anti- $H_2O_2$  defenses were provided mostly by a  $H_2O$  soluble fraction with a molecular weight in the range of 100 to 300 kDa. Although the plasma proteins ceruloplasmin and  $\alpha_1$ -antitrypsin can provide anti- $H_2O_2$  protection and are present in ELF, these macromolecules are in insufficient concentrations to provide the antioxidant protection observed in normal ELF. However, catalase, a normal intracellular antioxidant, was present in normal ELF in sufficient concentration to account for most of the observed anti- $H_2O_2$  properties of ELF. Furthermore, when ELF was depleted of catalase with an anti-catalase antibody, the anti- $H_2O_2$  macromolecular defenses of ELF were abolished. Although





the source of the catalase is unknown, it was not derived from red blood cells and evaluation of its concentration relative to other intracellular antioxidants and its isoenzyme pattern suggested that its presence was not due to an artifact of the lavage procedure and at least in part, cells other than inflammatory cells contribute to its presence. Since catalase is not normally released by cells, a likely explanation for its presence in high concentrations in normal ELF is that it is released by lung inflammatory and parenchymal cells onto the epithelial surface of the lower respiratory tract during their normal turnover and collects there due to the slow turnover of ELF. However, independent of its source or mechanisms of accumulation, it is likely that catalase in the ELF of the lower respiratory tract of normal individuals plays a role in protecting lung parenchymal cells against oxidants present in the extracellular milieu.

We have also evaluated ELF for the presence of glutathione (L- $\gamma$ -glutamyl-L-cysteinyl-glycine), another antioxidant that might contribute to the antioxidant protection of the epithelial surface of the lung. The theoretical basis for this hypothesis rests in the knowledge that, although glutathione is a major component of intracellular antioxidant defenses, it is exported extracellularly. Specifically, (1) among the cell types known to export glutathione are mononuclear phagocytes, lymphocytes and fibroblasts, cells present in the lower respiratory tract; (2) although peripheral blood plasma levels of glutathione are very low, the glutathione concentration in hepatic vein plasma is much higher, consistent with the concept that the concentrations of glutathione may vary at different sites in the body; (3)  $\gamma$ -glutamyl-transpeptidase, a cell surface enzyme that functions to remove glutathione from the extracellular space, is present in the lung, but in far lower concentrations in the lung parenchyma than in the kidney, the major site of glutathione removal from plasma; and (4) although the epithelial lining fluid of the lower respiratory tract is replenished constantly, this process is relatively sluggish so that materials released into this compartment likely remain in the local milieu for some time. Direct evaluation by lavage demonstrated the total glutathione (the reduced form GSH and the disulfide GSSG) concentration of normal ELF was 140-fold higher than that in plasma of the same individuals, and 96% of the glutathione in ELF was in the reduced form. Compared with nonsmokers, cigarette smokers had 80% higher levels of ELF total glutathione, 98% of which was in the reduced form. Studies of cultured lung epithelial cells and fibroblasts demonstrated that these concentrations of reduced glutathione were sufficient to protect these cells against the burden of  $H_2O_2$  in the range released by alveolar macrophages removed from the lower respiratory tract of nonsmokers and smokers, respectively, suggesting that the glutathione present in the alveolar ELF of normal individuals likely contributes to the protective screen against oxidants in the extracellular milieu of the lower respiratory tract.

The chronic inhalation of inorganic dust in an occupational setting can result in the development of interstitial lung disease, a chronic disorder of the lower respiratory tract associated with dyspnea and a limitation in the transfer of oxygen from air to blood. Referred to as "pneumoconioses," the common inorganic dust diseases are those resulting from the chronic exposure to high concentrations of airborne asbestos ("asbestosis"), coal ("coal workers' pneumoconiosis," CWP), and silica ("silicosis"). There has



been extensive study of the clinical manifestation of these disorders, and they are each well defined in terms of their associated symptomatology, physical findings, roentgenographic appearance, and physiologic dysfunction. However, despite the available epidemiologic and clinical information regarding these disorders, little is known of the processes occurring within the lower respiratory tract that are responsible for the derangements to the lung parenchyma that characterize these diseases. In this context, we assessed differences in cell populations and macrophage-derived mediator release within the lower respiratory tract of individuals with asbestosis, CWP, and silicosis. Specifically, we focused on the mediators being released by the chronic inflammatory cells in the lower respiratory tract that may play a role in mediating injury and fibrosis of the lung parenchyma in these disorders. In all 3 disorders, the inflammation was dominated by alveolar macrophages. Since a common feature of these interstitial lung diseases is concurrent injury and fibrosis of alveolar walls, we assessed whether these alveolar macrophages were spontaneously releasing mediators capable of giving rise to these changes. Alveolar macrophages from the study population were spontaneously releasing increased amounts of superoxide anion and hydrogen peroxide (both  $p < 0.01$  compared to normals), oxidants capable of injuring lung parenchymal cells. The alveolar macrophages were also spontaneously releasing significantly increased amounts of fibronectin and alveolar macrophage-derived growth factor (both  $p < 0.01$  compared to normals), mediators that act synergistically to signal fibroblast replication. Taken together, these findings define a major role for the alveolar macrophage in mediating the alveolar wall injury and fibrosis that characterize the common pneumoconioses, and suggest the alveolar macrophage is an important "target" for developing strategies designed to prevent loss of lung function in these individuals.

Chronic asbestos exposure is associated with the accumulation of mononuclear phagocytes in the lower respiratory tract. This process can be both protective and injurious, since macrophages can aid in asbestos clearance yet also modulate structural derangements of the alveolar walls. To understand why macrophages accumulate in the lungs of asbestos-exposed persons, two possible mechanisms were evaluated using alveolar macrophages from subjects with histories of chronic high exposure to airborne asbestos: (1) enhanced recruitment of blood monocytes to the lung; and (2) an increased rate of replication of macrophages *in situ*. Monoclonal antibody analysis with antibodies that detect surface antigens on the majority of circulating blood monocytes but only on a minority of mature alveolar macrophages demonstrated that an increased proportion of alveolar macrophages of asbestos workers expressed monocyte lineage antigens, suggesting the presence of "young" newly recruited macrophages and thus enhanced recruitment. Culture of the alveolar macrophages from these subjects with [ $^3\text{H}$ ]thymidine followed by autoradiography demonstrated an increased proportion of alveolar macrophages synthesizing DNA, suggesting the macrophages are replicating at an increased rate *in situ*. These observations are consistent with the concept that both enhanced recruitment of blood monocytes and increased local proliferation of alveolar macrophages contribute to the accumulation of mononuclear phagocytes to the lung of persons with chronic asbestos exposure.

Langerhans' cells represent a distinct population of bone marrow derived



cells associated with the mononuclear phagocyte system. Typically, these cells are 15 to 25  $\mu\text{m}$  in diameter, have a lobulated nucleus and, by ultrastructural analysis, demonstrate characteristic intracytoplasmic structures called Birbeck granules. Normally found in squamous epithelia such as the skin and in lymphoid organs such as the spleen, lymph nodes and thymus, Langerhans' cells are thought to function as antigen presenting cells. As such, Langerhans' cells are capable of phagocytosis, express HLA class II antigens and are capable of releasing interleukin-1. Langerhans' cells are relatively rare in the normal human lung, occasionally being found in the alveolar interstitium and bronchial walls; Langerhans' cells are not normally present on the pulmonary epithelial surface and are not normally found among the mononuclear phagocyte populations recovered by bronchoalveolar lavage. In marked contrast, Langerhans' cells occupy a prominent place among the cells accumulating in the lower respiratory tract in histiocytosis X, a disorder characterized by focal granulomatous lesions centered about the terminal bronchioles. Consistent with this knowledge, Langerhans' cells are commonly observed among the cells recovered by lavage of these patients. Although the etiology of pulmonary histiocytosis X is unknown, it is a striking epidemiologic observation that greater than 95% of affected individuals have a history of cigarette smoking. In this context, and with the knowledge that Langerhans' cells dominate the cellular lesions of histiocytosis X yet are very rare in the normal lung, we hypothesized that cigarette smoking may, in some manner, be associated with an expansion in the population of Langerhans' cells in the lung. To evaluate this hypothesis, we have examined cells recovered by bronchoalveolar lavage of normal nonsmokers and normal cigarette smokers for the presence of Langerhans' cells using both the monoclonal antibody OKT6, an antibody that recognizes Langerhans' cells, and transmission electron microscopy. While the OKT6 antibody identified  $0.1 \pm 0.1\%$  of the recovered cells in the nonsmokers, it labeled  $1.1 \pm 0.3\%$  recovered cells of the smokers ( $p < 0.01$ ). Furthermore, while electron microscopy demonstrated no Langerhans' cells among the lavage cells from nonsmokers,  $0.4 \pm 0.1\%$  of the cells recovered from normal smokers contained characteristic intracytoplasmic Birbeck granules, positively identifying them as Langerhans' cells. We conclude that cigarette smoking is associated with an expansion in the population of Langerhans' cells on the epithelial surface of the lower respiratory tract. While the mechanisms underlying this accumulation are unknown, it is possible that the properties of these cells contribute to the derangements of the pulmonary parenchyma found in cigarette smoking and establish a biological link to the already observed epidemiological association between histiocytosis X and cigarette smoking.

## II. Disorders Characterized by the Accumulation of T-Lymphocytes in the Lower Respiratory Tract.

Pulmonary sarcoidosis is a disorder of the lower respiratory tract characterized by chronic inflammation, granuloma formation and, in some individuals, parenchymal fibrosis. Together these processes derange the alveoli, airways and blood vessels, consequently impairing the ability of the lung to exchange gas in the normal fashion. As with the other interstitial lung disorders, it is recognized that the inflammation precedes the other abnormalities that characterize this disorder. The inflammation of active pulmonary sarcoid is dominated by an accumulation of T-helper lymphocytes



in the lung parenchyma. These T-cells are thought to play a central role in the pathogenesis of sarcoidosis in two ways. First, the accumulated T-cells distort the architecture of the parenchyma, thus altering the intimate relationships between air and blood. Second, the T-cell populations are activated and spontaneously releasing monocyte chemotactic factor and interferon-gamma, mediators that recruit and activate mononuclear phagocytes, respectively, events that are early steps in the process of granuloma formation. In this context, an understanding of the pathogenesis of pulmonary sarcoidosis is intimately linked to the understanding of the process directing the accumulation of T-lymphocytes in the lower respiratory tract of individuals with active disease. Relevant to this question, prior work from this laboratory has demonstrated that the T-lymphocytes recovered from the lungs of these patients are spontaneously proliferating and spontaneously releasing interleukin-2 (IL-2), the T-cell growth factor. Further analysis demonstrated that it is the activated HLA-DR+ helper (CD4+) T-cells which release the majority of the IL-2. Thus, while the stimulus that initiates the process is unknown, the IL-2 releasing lung T-cells are thought to be responsible for maintaining the T-cell inflammation and thus maintaining the disease in an active state.

To determine whether the IL-2 gene is activated in sarcoid T-cells in a systemic manner or only at sites of disease, cells obtained by bronchoalveolar lavage of individuals with active sarcoidosis, inactive sarcoidosis, and normals were evaluated for the spontaneous presence of IL-2 transcripts by using a human IL-2 cDNA probe and Northern analysis of extracted RNA. Freshly recovered lung cells of individuals with active pulmonary sarcoidosis contained 0.85 kb IL-2 mRNA transcripts. However, IL-2 mRNA transcripts could not be detected in fresh autologous blood T-cells or in purified autologous blood CD4+ T-cells, although IL-2 mRNA transcripts were inducible in these cells by phytohemagglutinin/phorbol myristate acetate. The sarcoid lung T cells, however, did not express the IL-2 gene constitutively; when placed in culture with no stimulation and evaluated after 24 hr, they demonstrated down regulation of the amounts of IL-2 mRNA transcripts, despite the fact that they were capable of re-expressing the IL-2 gene and releasing more IL-2 in response to added activation signals. Thus the activation of the IL-2 gene in T-cells in active sarcoidosis occurs at the site of disease and is not a generalized property of T-cells throughout the body, and is not sustained if the T-cells are removed from the sites of disease. Although the cause of sarcoid is unknown, these observations are consistent with the concept that sarcoid is associated with local stimuli at the site of disease inducing the CD4+ T cell IL-2 gene activation that plays such a critical role in the pathogenesis of this disease.

Since active pulmonary sarcoidosis is characterized by the alveolar accumulation of activated helper T-lymphocytes which are spontaneously releasing IL-2 and proliferating at an enhanced rate, sarcoidosis represents a "model" human disorder to test in vivo the known in vitro action of corticosteroids on suppressing the activated IL-2 gene. To evaluate this concept, comparable groups of patients with active sarcoidosis were prospectively evaluated with no therapy or treated with corticosteroids. Over  $3.2 \pm 0.4$  months, the untreated group had no significant change in spontaneous lung T-cell release of IL-2 or spontaneous proliferation. In contrast, over the same period, the treated group had marked reduction of





spontaneous lung T-cell release of IL-2 and proliferation ( $p < 0.01$ , all comparisons prior to therapy). Furthermore, Northern analysis of lung T-cell RNA prior to therapy demonstrated IL-2 mRNA transcripts while no IL-2 transcripts were observed during therapy. These observations are consistent with the concept that directly, or indirectly, corticosteroids are capable of suppressing the IL-2 gene in activated T-lymphocytes in vivo.

The exaggerated T-helper/inducer processes observed in sarcoid are in striking contrast to normal cellular immune responses, in which the initial accumulation of T-helper/inducer cells is followed by accumulation of T-suppressor/cytotoxic cells, a process associated with the damping of the overall immune response. In this regard, the exuberant T-helper/inducer processes in sarcoidosis could be explained by hypothesizing the presence of stimuli specific for helper/inducer T-cells that do not stimulate suppressor/cytotoxic T-cells and/or processes that specifically suppress suppressor/cytotoxic T-cells. Relevant to these concepts, it is known that individuals with sarcoidosis have circulating anti-T-cell antibodies, primarily of the IgM class. In this context, and in the context that active pulmonary sarcoidosis is characterized by the activation of T-helper/inducer cells in the lung without a concomitant increase in T-suppressor/cytotoxic cells, we evaluated these antibodies for a possible functional role in the pathogenesis of sarcoidosis. The strategy to examine this question was to evaluate serum and lavage fluid of active sarcoid patients for the presence of anti-T-lymphocyte antibodies, identify the target T-cell subset for these antibodies and evaluate these antibodies for possible stimulatory effects on T-helper/inducer cells and/or inhibitory effects on T-suppressor/cytotoxic cells. Indirect immunofluorescence studies demonstrated that sarcoid patients had anti-T-cell antibodies of the IgM type reacting with autologous as well as non-autologous normal T-cells. IgM recovered in sarcoid lavage fluid also reacted with T-cells, thus demonstrating the autoantibodies at the site of disease. Two color immunofluorescence and flow cytometry showed that these sarcoid autoantibodies bound to mostly CD8+ T-suppressor/cytotoxic cells, but also to a small proportion of CD4+ T-helper/inducer cells. Incubating lymphocytes with sarcoid serum or IgM purified from sarcoid serum did not stimulate T-cell proliferation. Furthermore, when CD8+ T-cells were stimulated with irradiated allogenic B-cells, increasing concentrations of sarcoid serum had no inhibitory effects on the activation and proliferative response of the CD8+ T-cells. Likewise, the purified IgM anti-T-cell antibodies had no inhibitory effects on the mitogenic response of CD8+ T-cells to the anti-T-cell antigen receptor-associated T3 complex antibody OKT3. Thus, the IgM anti-T-cell autoantibodies of sarcoidosis are present at the site of disease and bind to potentially relevant T-cell subsets, but there is no evidence that they have an identifiable functional role in the development of the excess T-helper cell activity of sarcoidosis.

One clue to the mechanisms responsible for the exaggerated CD4+ T-cell inflammatory process in the sarcoid lung relates to the observation that the activation of lung CD4+ T-cell population occurs in the absence of comparable activation of lung CD8+ T-cells. In this context, we hypothesized that CD8+ T-suppressor/cytotoxic cells in sarcoid may have an impaired ability to respond to activation stimuli and to generate effector cells



capable of suppressing the activation and proliferation of the helper/inducer T-cell population at the site of disease. If this occurs, it would help to explain why the spontaneous down-regulation of activated T-helper/inducer processes that characterizes normal immune responses is not observed in active pulmonary sarcoidosis. To evaluate this concept, a study was designed to assess the response of sarcoid CD8+ T-cells for their ability to express IL-2 receptors and proliferate in response to an activation signal and the ability of antigen activated CD4+ T-cells to activate autologous CD8+ T-cells to function to suppress antigen-specific CD4+ T-cell proliferation. Stimulation of purified sarcoid blood CD8+ T cells with the anti-T3/Ti complex monoclonal antibody OKT3 was followed by the normal expression of IL-2 receptor ( $p > 0.1$ ) and proliferation ( $p > 0.1$ ). In addition, lung sarcoid CD8+ T-cells responded to OKT3 similarly to autologous lung CD4+ T-cells as regards expression of IL-2 receptor ( $p > 0.1$ ) and proliferation ( $p > 0.1$ ). Finally, using CD4+ cells activated with allogenic antigen to induce, in co-culture, fresh autologous CD8+ cells to suppress proliferation of fresh autologous CD4+ cells to the same antigens, sarcoid CD8+ T-cells suppressed CD4+ cell proliferation in a normal fashion ( $p > 0.1$ ). These results demonstrate that sarcoid CD8+ (suppressor/cytotoxic) T-cells are competent to respond to a proliferation signal normally and can be induced to normally suppress CD4+ T-cell proliferation to antigens, suggesting that the expansion of activated CD4+ T-cells in pulmonary sarcoidosis is neither due to a generalized abnormality of CD8+ T-cells nor of their suppressor T-cell function.

One mechanism to explain the activated T-cell processes in sarcoid is to hypothesize that the T-cells in affected organs are "driven" to be activated in an exaggerated fashion by mononuclear phagocytes in the local environs, i.e., that sarcoid is associated with abnormalities of mononuclear phagocytes, in which the mononuclear phagocytes function in an enhanced fashion in their interactions with T-lymphocytes. Consistent with this concept, alveolar macrophages of individuals with sarcoidosis present antigen in an exaggerated fashion to autologous blood and lung T-lymphocytes as well as to HLA-DR-matched T-cell lines. In this regard, it is conceivable that sarcoid results, at least in part, from the heightened response of mononuclear cells to antigens localized at sites of disease. If this hypothesis is correct, it should be possible to identify those genes responsible for the putative hypermacrophagic state. One candidate gene is that coding for interleukin- $1\beta$  (IL- $1\beta$ ), a 17 kDa protein that is the dominant species of interleukin-1 produced by human mononuclear phagocytes. Relevant to the pathogenesis of sarcoidosis, it has been suggested that alveolar macrophages of patients with active sarcoid are spontaneously releasing IL-1, while alveolar macrophages of normal individual are not. If correct, this would help explain why these individuals have exaggerated numbers of activated proliferating T-cells in the lower respiratory tract. In the context of these considerations, a study was designed to evaluate the expression of the IL- $1\beta$  gene in alveolar macrophages obtained from the lungs of individuals with active pulmonary sarcoidosis. Evaluation of media from unstimulated cultured sarcoid alveolar macrophages failed to detect IL-1 activity. When parallel cultures of sarcoid and normal alveolar macrophages were stimulated with lipopolysaccharide (LPS), they released similar amounts of IL-1 activity. Using a highly specific polyclonal anti-IL- $1\beta$  antibody and flow cytometry to evaluate cell-associated



IL-1 $\beta$ , analysis of fresh alveolar macrophages from patients with active sarcoidosis and normal individuals revealed no detectable cell-associated IL-1 $\beta$ , but IL-1 $\beta$  was present when macrophages from sarcoid patients and normals were stimulated with LPS. Similar observations were made using immunoblot analysis of cell lysates of the same unstimulated and stimulated macrophages. Finally, Northern analysis of alveolar macrophages for IL-1 $\beta$  mRNA transcripts demonstrated minimal, but equivalent, amounts of IL-1 $\beta$  in both normal and sarcoid macrophages, as compared to the much larger quantities present in LPS-stimulated alveolar macrophages. Thus, while alveolar macrophages of individuals with sarcoidosis are clearly capable of expressing the IL-1 $\beta$  gene, these findings suggest that altered expression of the IL-1 $\beta$  gene by alveolar macrophages does not play a central role in the exaggerated lung T-cell activation characteristic of sarcoidosis.

To evaluate the possible mechanisms underlying the observation that mature populations of human mononuclear phagocytes are relatively poor IL-1 producers compared to blood monocytes, the expression of the IL-1 $\beta$  gene mRNA transcripts was quantified in LPS-stimulated normal autologous blood monocytes and alveolar macrophages using a labeled IL-1 $\beta$  cDNA probe. Although Northern analysis demonstrated that stimulated monocytes and alveolar macrophages both express 1.8 kb IL-1 $\beta$  mRNA transcripts, cytoplasmic dot blot analysis showed that the total IL-1 $\beta$  mRNA content in alveolar macrophages was only 38 $\pm$ 5% of that in blood monocytes after LPS-stimulation. Interestingly, *in situ* hybridization using antisense and sense IL-1 $\beta$  RNA probes revealed that whereas most of stimulated blood monocytes contained IL-1 $\beta$  mRNA transcripts, a significant proportion of autologous alveolar macrophages stimulated in an identical fashion did not express the IL-1 $\beta$  gene. In this regard, within 4 hr, 81 $\pm$ 6% LPS stimulated monocytes contained IL-1 $\beta$  mRNA transcripts. In contrast, 4 hr after stimulation, 16 $\pm$ 9% of alveolar macrophages contained IL-1 $\beta$  transcripts and by 18 hr, this had only increased to 43 $\pm$ 15%. Quantification of the size distribution of the IL-1 $\beta$  mRNA expressing monocytes and alveolar macrophages demonstrated that the majority of monocytes expressing the IL-1 $\beta$  gene were 10 to 15  $\mu$ m whereas the majority of alveolar macrophages were 10 to 25  $\mu$ m i.e., among the population of alveolar macrophages, the cells expressing this gene were not confined to those that were "monocyte-like". Together, these observations suggest that there is a significant heterogeneity among populations of mononuclear phagocytes in their ability to express the gene for IL-1 $\beta$  in response to a standard stimulus, and this heterogeneity explains, in part, the observation that differentiated forms of mononuclear phagocytes such as alveolar macrophages are relatively poor IL-1 producers.

A central question is understanding the pathogenesis of sarcoidosis is to understand why T-lymphocytes accumulate at sites of disease such as the lower respiratory tract. Three general hypotheses can be proposed to explain the pathogenesis of these T-lymphocyte infiltrations in sarcoidosis. First, the accumulated T-cells may represent a polyclonal T-cell response, perhaps secondary to a generalized enhancement of T-helper cell processes or ineffective T-cell suppressor networks that are not antigen-specific. Second, the T-cells may accumulate secondary to a monoclonal or oligoclonal process such as that observed in malignancies in which a "transformed" cell with a growth advantage accumulates in tissues. Third, the T-cells may accumulate secondary to antigen-driven processes, in which



one or several antigens drive the clonal expansion of antigen-specific T-cells together with the secondary expansion of populations of immunoregulatory and/or bystander T-cells. Since the mechanisms responsible for T-cell accumulation in these three categories are different, an understanding of whether the lung T-cells are polyclonal, oligoclonal, or monoclonal is an important step in understanding the pathogenesis of this disorder. As an approach to this question, we have capitalized on the recent identification and cloning of the  $\beta$ -chain gene of the T-cell antigen receptor. Since the  $\beta$ -chain gene of the T-cell antigen receptor undergoes specific DNA rearrangement during normal T-cell ontogeny, these rearrangements serve as a marker for clonal populations of T-cells that can be detected by Southern blot analysis of DNA isolated from T-cells. Using this immunogenotypic approach, we evaluated T-cells from the lung and blood of patients with active sarcoidosis, inactive sarcoidosis and normals. DNA from lung T-cells of 7 of 10 individuals with active sarcoidosis demonstrated non-germline bands on Southern blot analysis using a  $\beta$ -chain gene constant region probe, consistent with the presence of T-cells with "clonal" rearrangements of the  $\beta$ -chain gene locus. In contrast, normal individuals and 5 of 5 cases of inactive pulmonary sarcoidosis had no detectable  $\beta$ -chain gene rearrangements among their lung T-cells. Interestingly, blood T-cell DNA of 6 of 10 individuals with active pulmonary sarcoidosis also demonstrated clonal rearrangements of the  $\beta$ -chain gene, indicating a systemic nature of the T-cell processes associated with this disorder. The  $\beta$ -chain rearrangements of lung and blood T-cells of different individuals with sarcoidosis persisted over many months. Together, these observations suggest that a population of T-cells with a highly restricted repertoire of  $\beta$ -chain gene rearrangements are involved in the chronic inflammatory processes that characterize this disorder.

Current concepts of the pathogenesis of sarcoidosis suggest that the expanded numbers of activated T-helper/inducer cells at sites of disease activity result, at least in part, from their proliferation in the local milieu. Since normal clonal proliferation of T-cells involves activation and expression of the IL-2 receptor and its subsequent interaction with IL-2, we evaluated these concepts by quantifying lung and blood IL-2 receptor gene mRNA transcripts and IL-2 receptor cell surface protein of patients with active and inactive sarcoidosis and normals. Northern analysis of RNA extracted from lung T-cells using a  $^{32}\text{P}$ -labeled IL-2 receptor cDNA probe demonstrated that patients with active sarcoidosis express 3.5 kb and 1.5 kb IL-2 receptor mRNA transcripts, the same as observed in normal T-cells activated in vitro. Consistent with this, using flow cytometry and a monoclonal antibody directed against the IL-2 receptor protein (2A3), significant levels of IL-2 receptor protein were observed on the surface of both lung and blood T-cells of active sarcoidosis patients (lung  $5.7 \pm 2.4\%$ , blood  $5.8 \pm 1.7\%$ ). In contrast, while normal lung T-cells expressed the IL-2 receptor surface protein ( $2.9 \pm 1.0\%$ ), normal blood T-cells did not contain detectable IL-2 receptor mRNA and IL-2 receptor protein was detectable very rarely ( $0.5 \pm 0.4\%$ ). Thus, sarcoidosis is associated with both tissue and circulating T-cells expressing the IL-2 receptor gene. When placed in the context of the known compartmentalization of T-cell proliferation and spontaneous IL-2 production in active pulmonary sarcoidosis, it is possible that these IL-2 receptor positive cells represent a subset of T-cells that have a proliferative advantage





when present in a local milieu with IL-2 present, such as the lower respiratory tract of patients with active pulmonary sarcoidosis.

Pulmonary berylliosis is a chronic granulomatous disorder characterized by accumulation of helper T-cells, macrophages, and granulomas in the lower respiratory tract. To evaluate the hypothesis that the expansion of large numbers of CD4+ T-cells in the lung of patients with pulmonary berylliosis is associated with Be-specific CD4+ T-cell activation, the response to Be of lung T-cells was evaluated in four patients with pulmonary berylliosis. All had abnormal chest X-rays and gallium scans, increased proportions of lung lymphocytes ( $49 \pm 5$ ; normal  $12 \pm 2$ ;  $p < 0.001$ ), increased helper/suppressor ratios ( $7 \pm 4$ , normal  $2 \pm 1$ ,  $p < 0.5$ ) and high proportions of activated (DR+) CD4+ lung T-cells ( $61 \pm 5$  of CD4+, normal  $7 \pm 1\%$ ,  $p < 0.001$ ). To show that the Be-induced T-cell proliferation is HLA-DR restricted, and requires the activation of interleukin-2 receptor (IL2R) pathway, purified lung T-cells + 20% monocytes were cultured with BeSO<sub>4</sub> ( $0.5 \times 10^{-5}$  -  $0.5 \times 10^{-4}$ M), with or without the anti-HLA-DR monoclonal antibody 9.49 or the anti-IL2R antibody anti-Tac, and pulsed with <sup>3</sup>H-thymidine after 5 day culture. T-cell proliferation to BeSO<sub>4</sub> (stimulation index  $79 \pm 29$ ) was significantly prevented by anti-DR ( $97 \pm 1\%$  inhibition) and by anti-IL2R ( $83 \pm 4\%$  inhibition). To determine which lung T-cell subset proliferated in response to Be, purified lung CD4+ or CD8+ T-cells were cultured with 20% monocytes. While CD4+ lung T-cells proliferated in response to BeSO<sub>4</sub> (stimulation index  $102 \pm 33$ ), CD8+ lung T-cells did not ( $2 \pm 1$ ,  $p < 0.5$  compared to CD4+). In addition, flow cytometry propidium iodide cell cycle analysis of unfract ionated lung mononuclear cells cultured with Be for 5 days demonstrated that  $10.8 \pm 2.1\%$  of CD4+ cells were in S,G<sub>2</sub>,M phases of cell cycle compared to  $3.4 \pm 0.9\%$  CD8+ cells ( $p < 0.05$ ). Thus, in individuals with berylliosis, lung T-cells respond to Be with antigen specific, HLA-D-restricted, IL2/IL2R dependent activation of CD4+ T-cells, suggesting this process plays a fundamental role in expanding the helper T-cell population in the lungs in this disorder.

T-lymphocytes on the epithelial surface of the lower respiratory tract are thought to represent a relatively compartmentalized population of T-cells that exchanges slowly with the blood. Since the lung is chronically burdened with antigens, "resident" T-cells likely have a history of past activation. To evaluate this concept, we analyzed resident lung T-cells for VLA-1 expression, which is indicative of a history of past stimulation. Lung lavage and blood T-cells were evaluated in 13 normal nonsmokers using the monoclonal antibodies Leu4 (CD3, pan T-cells), Leu3 (CD4, helper/inducer T-cells), Leu2 (CD8, suppressor/cytotoxic T-cells), TS2/7 ( $\alpha 1$  subunit of VLA-1) and A-1A5 ( $\beta$  subunit of VLA-1) using immunofluorescence and immunoprecipitation. In contrast to normal blood T-cells which did not express VLA-1, lung T-cells expressed the 210 kDa  $\alpha 1$  and 130 kDa  $\beta$  subunits of the VLA-1 complex, the same as blood T-cells activated in culture for three weeks. Two color immunofluorescence with Leu4 and TS2/7 showed that  $19 \pm 6\%$  of the lung T-cells were VLA-1+, suggesting that a significant proportion of T-lymphocytes on the alveolar epithelial surface are in a separate compartment from the VLA-1 blood cells. In sarcoidosis, a disease characterized by exaggerated numbers of active CD4+ T-cells in the lower respiratory tract, increased numbers of lung CD4+ T-cells expressing VLA-1 were present on the epithelial surface of the lung ( $p < 0.05$  compared to normals).



These observations are consistent with compartmentalized, chronically stimulated T-lymphocytes on the alveolar epithelial surface that exchange with the systemic immune system very slowly.

### III. Disorders Characterized by Destruction of the Alveolar Walls

Alpha 1-antitrypsin ( $\alpha$ 1AT), a 52,000 dalton serum glycoprotein produced by hepatocytes and mononuclear phagocytes, functions to inhibit neutrophil elastase, a proteolytic enzyme capable of destroying all protein components of connective tissue. The  $\alpha$ 1AT gene is highly pleomorphic; more than 30 different alleles have been described. The  $\alpha$ 1AT phenotype, referred to as the Pi (protease inhibitor) type, represents the codominant expression of the two parental  $\alpha$ 1AT alleles. The most common  $\alpha$ 1AT alleles in the U.S.A. are those of the M-family (combined frequency greater than 0.90), the S type (frequency 0.02-0.04) and the Z type (0.01-0.02). The clinical interest in these  $\alpha$ 1AT alleles is based on the knowledge that inheritance of the phenotypes PiZZ and PiSZ are associated with an increased risk for the development of emphysema (in adults) and/or liver disease (in children).

In the context that Pulmonary Branch, NHLBI, is a national referral center for adults in the USA with symptomatic emphysema with  $\alpha$ 1AT deficiency, we evaluated 124 such individuals using a variety of routine clinical parameters in order to draw conclusions concerning the characteristics of such a population. The typical adult  $\alpha$ 1AT deficient patient with symptomatic emphysema has an  $\alpha$ 1AT level of 30 mg/dl, and almost always has the PiZZ phenotype. This individual was most often male, Caucasian, an ex-smoker and became dyspneic at ages 25 to 40. Most routine blood tests were normal. The chest X-ray and ventilation-perfusion studies typically demonstrated abnormalities with a lower zone distribution and about one-third had evidence suggestive of pulmonary hypertension. Lung function tests were typical for emphysema. The FEV<sub>1</sub> and DLCO were the parameters most dramatically reduced, and the annual rate of decline of those parameters was greater than that of the general population. The cumulative probability of survival of PiZZ deficient adults with pulmonary symptoms indicated a significantly shortened lifespan with a mean survival of 16% at age 60 compared with 85% for normal individuals.

The emphysema of  $\alpha$ 1AT deficiency is conceptualized to result from insufficient  $\alpha$ 1AT allowing neutrophil elastase (NE) to work unimpeded to destroy lung parenchyma. Lavage studies have shown that  $\alpha$ 1AT deficient individuals have increased numbers of neutrophils (PMN) in their lungs compared to normal individuals. With the knowledge that alveolar macrophages (AM) have surface receptors for NE, we hypothesized that PMN accumulation in lung parenchyma in  $\alpha$ 1AT deficiency may result from release of PMN chemotactic activity by AM as they bind and internalize uninhibited NE. To evaluate this hypothesis, we compared spontaneous PMN chemotactic activity release by AM from  $\alpha$ 1AT deficient individuals to that by AM of normals using a conventional chemotactic assay with human PMN as the responding cells.  $\alpha$ 1AT deficient AM spontaneously released more than 3-fold as much PMN chemotactic activity as normal AM ( $\alpha$ 1AT deficient  $305 \pm 63$  U/ $10^6$  AM-3 hr vs normal  $82 \pm 94$ ,  $p < .01$ ). Consistent with this hypothesis, the incubation of normal AM with NE resulted in an NE dose dependent increase in PMN chemotactic activity release (100nM NE, 37°:  $384 \pm 90$  U/ $10^6$  AM-3 hr,  $p < .01$



vs non-NE exposed AM), similar to the level seen following incubation of normal AM with zymosan ( $439 \pm 92$  U/ $10^6$  AM-3 hr). However, preincubation of NE with  $\alpha$ 1AT prior to its addition to normal AM led to an  $\alpha$ 1AT dose-dependent decrease in release of PMN chemotactic activity. HPLC and molecular sieve chromatography demonstrated that the PMN chemotactic signal(s) released by  $\alpha$ 1AT deficient AM and NE stimulated normal AM included leukotriene B<sub>4</sub> (LTB<sub>4</sub>). These results suggest that a mechanism for the increased PMN in the lungs of  $\alpha$ 1AT deficient individuals may be the release of LTB<sub>4</sub> by AM as they bind free elastase. In the  $\alpha$ 1AT deficient lung this may establish a cycle in which non- $\alpha$ 1AT bound NE is taken up by AM, which then release LTB<sub>4</sub>, causing the recruitment of still more PMN, with their elastase. Thus, a chronically increased PMN number and NE burden is maintained, leading to accelerated lung destruction in  $\alpha$ 1AT deficiency.

The entire nucleotide sequence of the protein coding region sequence of the  $\alpha$ 1AT Z gene was determined using conventional cloning methods. In addition to the glu<sup>342</sup> to lys<sup>342</sup> mutation in exon V which has been previously identified by peptide analysis, another point mutation (GTG to GCG in exon III) in the gene sequence predicts a second amino acid substitution (val<sup>213</sup> to ala<sup>213</sup>) in the Z protein. This val<sup>213</sup> to ala<sup>213</sup> mutation was confirmed to be a general finding in Z type  $\alpha$ 1AT gene by evaluating genomic DNA from 40 Z alleles using synthetic oligonucleotide gene probes directed toward the mutated exon III sequences in the Z gene. Furthermore, the exon III val<sup>213</sup> to ala<sup>213</sup> mutation eliminates a BstEII restriction endonuclease site in the  $\alpha$ 1AT Z gene, allowing rapid identification of this val<sup>213</sup> to ala<sup>213</sup> substitution at the genomic DNA level. Surprisingly, when genomic DNA samples from individuals thought to be homozygous for the M1 gene (the most common  $\alpha$ 1AT normal allele) were evaluated with BstEII, 23% of the M1 alleles were BstEII site negative, thus identifying a new form of M1 (i.e., M1(ala<sup>213</sup>)), likely identical to M1 but with an isoelectric focusing "silent" amino acid substitution (val<sup>213</sup> to ala<sup>213</sup>). Although the relative importance of the newly identified exon III val<sup>213</sup> to ala<sup>213</sup> mutation to the pathogenesis of the abnormalities associated with the Z gene is not known, it is likely that M1(Ala<sup>213</sup>) gene represents a common "normal" polymorphism of the  $\alpha$ 1AT gene that served as an evolutionary intermediate between the M1(Val<sup>213</sup>) and Z genes.

In normal individuals with the homozygous inheritance of the common M1-type  $\alpha$ 1AT gene, the serum  $\alpha$ 1AT levels are 150 to 350 mg/dl. In contrast, in the common form of  $\alpha$ 1AT deficiency associated with homozygous inheritance of the Z-type  $\alpha$ 1AT gene, serum  $\alpha$ 1AT levels are invariably less than 50 mg/dl. In this context, it is accepted that the reduction of  $\alpha$ 1AT levels to <50 mg/dl is sufficient to permit the burden of neutrophils in the lower respiratory tract to slowly destroy the lung parenchyma. However, while this logic gives a compelling basis for understanding the pathogenesis of the emphysema associated with  $\alpha$ 1AT deficiency, it ignores the data from epidemiologic studies that has convincingly shown that while serum  $\alpha$ 1AT levels of 150 mg/dl are the lower limit of normal individuals, individuals with levels of  $\geq 80$  mg/dl are at no increased risk for the development of emphysema beyond the risk for the general population. Thus, while individuals with homozygous inheritance of the Z gene commonly have  $\alpha$ 1AT levels in the 25 to 45 mg/dl range, those with only 2 to 2.5-fold



more  $\alpha$ 1AT have no increased risk for emphysema, i.e., the epidemiologic data leads to the conclusion that a relatively small reduction in  $\alpha$ 1AT levels is sufficient to place the individual at high risk for the development of emphysema. While such a reduction in the anti-neutrophil elastase screen for the lower respiratory tract could be sufficient to cause such a high risk for disease, we have hypothesized that the mutations in the coding sequence for the  $\alpha$ 1AT gene may result, in addition to a reduction in the serum levels of  $\alpha$ 1AT, in a diminished ability of the  $\alpha$ 1AT molecule to work effectively as an inhibitor of neutrophil elastase. In this context, a study was designed to evaluate the concept that, in addition to the Z-type  $\alpha$ 1AT protein being present in reduced amounts, the molecule itself is less able to inhibit neutrophil elastase compared to the normal M1 molecule. To evaluate this hypothesis, the functional status of  $\alpha$ 1AT from PiZZ individuals was compared with that of  $\alpha$ 1AT from PiM1M1 individuals for its ability to inhibit neutrophil elastase (% inhibition) as well as its association rate constant for neutrophil elastase ( $K$  association). Plasma  $\alpha$ 1AT concentration, measured by radial immunodiffusion, was  $34 \pm 1$  mg/dl in PiZZ patients vs  $237 \pm 14$  mg/dl for PiM1M1 plasma, a 7-fold difference. When titrated against neutrophil elastase, the percent inhibition of PiZZ plasma was significantly less than Pi M1M1 plasma (ZZ  $78 \pm 1\%$  vs M1M1  $95 \pm 1\%$ ,  $p < 0.001$ ) as was purified Z type  $\alpha$ 1AT (ZZ,  $63 \pm 2\%$  vs M1M1  $86 \pm 2\%$ ,  $p < 0.001$ ). SDS gel comparisons of the complexes formed with M1-type  $\alpha$ 1AT and Z-type  $\alpha$ 1AT with elastase demonstrated that the Z-type  $\alpha$ 1AT elastase complexes were less stable than the M1-type  $\alpha$ 1AT-elastase complexes, thus releasing some of the enzyme to continue to function as a protease. Consistent with these observations, the  $K$  association of purified Z-type  $\alpha$ 1AT for neutrophil elastase was lower than that of M1-type  $\alpha$ 1AT (ZZ  $4.5 \pm 0.3 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$  vs M1M1  $9.7 \pm 0.4 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ ,  $p < 0.001$ ), suggesting that for the population of  $\alpha$ 1AT molecules, the active Z-type molecules take more than twice as long as the active M1-type  $\alpha$ 1AT to inhibit neutrophil elastase. Consequently, not only is there less  $\alpha$ 1AT in PiZZ individuals, but the population of Z-type  $\alpha$ 1AT molecules are less competent as inhibitors of neutrophil elastase than M1-type  $\alpha$ 1AT molecules. This combination of defects suggests that PiZZ individuals have far less functional anti-elastase protection than suggested by the reduced concentrations of  $\alpha$ 1AT alone, further explaining their profound risk for development of emphysema.

In the course of studying the  $\alpha$ 1AT type Z gene, we identified a restriction endonuclease BstEII polymorphism in the M1 gene that predicted the existence of a previously unidentified, but relatively common allele of M, referred to as M1(ala<sup>213</sup>). We cloned both  $\alpha$ 1AT genes from an individual heterozygous for the M1(ala<sup>213</sup>) and M1(val<sup>213</sup>) alleles. Sequencing of the coding exons of both demonstrated that they are identical except for the ala-val difference at residue 213. The codominant transmission of the M1(ala<sup>213</sup>) gene was demonstrated in a family study. Evaluation of 39 genomic samples of Caucasians with the IEF allele M1 demonstrated allelic frequencies 68% for M1(val<sup>213</sup>) and 32% for M1(ala<sup>213</sup>).  $\alpha$ 1AT serum levels of individuals inheriting the M1 (ala<sup>213</sup>) gene in a homozygous fashion were in the same range as those for homozygous M1(val<sup>213</sup>) as was the rate of association of the M1(ala<sup>213</sup>) protein with neutrophil elastase. Interestingly, comparison of the M1(ala<sup>213</sup>) gene sequence to all of the known  $\alpha$ 1AT sequences at the gene and protein levels demonstrated that M1(ala<sup>213</sup>) is the closest to the baboon  $\alpha$ 1AT coding exons, suggesting that M1(ala<sup>213</sup>) is





the "oldest" type human  $\alpha$ 1AT known.

Sequencing of the Z  $\alpha$ 1AT gene has demonstrated it differs from the most common normal form of  $\alpha$ 1AT [ $M_1(\text{val}^{213})$ ] in two residues,  $\text{val}^{213} \rightarrow \text{ala}$  and  $\text{glu}^{342} \rightarrow \text{lys}$ . Since the 342 residue participates in a salt bridge ( $\text{glu}^{342}$ ,  $\text{lys}^{290}$ ) in the 3-dimensional structure of the protein, it has been suggested that the 342 mutation causes a disruption of the salt bridge resulting in the intracellular accumulation and reduced secretion of the Z protein by the  $\alpha$ 1AT producing cells. To directly prove this concept we modified the  $M_1(\text{val}^{213})$  full length  $\alpha$ 1AT human cDNA by oligonucleotide directed mutagenesis utilizing the single strand DNA bacteriophage M13 and a 19 base oligonucleotide to change the sequence to  $M_1(\text{val}^{213}, \text{glu}^{342} \rightarrow \text{lys})$  (i.e., the  $\text{glu}^{342} \rightarrow \text{lys}$  mutation on the  $M_1(\text{val}^{213})$  background). In parallel with the normal  $M_1(\text{val}^{213})$  cDNA, this cDNA was placed in the expression vector pSVL, which allows for gene expression in mammalian cells. Using calcium phosphate DNA transfection, log phase Cos I cells (SV40 virus transformed green monkey kidney cells) were transfected with equal amounts of DNA of either the mutated or normal  $\alpha$ 1AT sequence. RNA harvested at 72 hr and analyzed using a  $^{32}\text{P}$ -labeled  $\alpha$ 1AT cDNA probe demonstrated the  $M_1(\text{val}^{213})$  and  $M_1(\text{val}^{213}, \text{glu}^{342} \rightarrow \text{lys})$  transfected cultures produced similar amounts of  $\alpha$ 1AT mRNA. However, when the transfected cells were labeled with [ $^{35}\text{S}$ ]methionine in a pulse-chase fashion and the secreted  $\alpha$ 1AT was analyzed by immunoprecipitation with anti- $\alpha$ 1-antitrypsin antibody, SDS PAGE and autoradiography, the amount of  $\alpha$ 1AT secreted by  $M_1(\text{val}^{213}, \text{glu}^{342} \rightarrow \text{lys})$  transfected cultures was  $7 \pm 1$  fold less ( $p < 0.01$ ) than that of the  $M_1(\text{val}^{213})$  transfected cultures. Thus, even on a  $M_1(\text{val}^{213})$  background a single base alteration of G-A at codon 342 clearly causes a marked reduction in the amount of secreted  $\alpha$ 1AT similar to that seen in Z homozygotes.

S-type  $\alpha$ 1-antitrypsin ( $\alpha$ 1AT) is a deficiency allele that differs from the common normal  $M_1(\text{val}^{213})$   $\alpha$ 1AT allele by a single amino acid ( $\text{glu}^{264} \rightarrow \text{val}^{264}$ ). To evaluate the adequacy of the anti-neutrophil elastase protection associated with the S homozygous state,  $\alpha$ 1AT plasma and lung epithelial lining fluid (ELF) levels and anti-neutrophil elastase function were analyzed in PiSS individuals. The plasma  $\alpha$ 1AT levels of SS individuals were intermediate between that of  $M_1M_1$  and ZZ individuals ( $p < 0.001$  all comparisons) and the plasma neutrophil elastase inhibitory capacity paralleled the differences in  $\alpha$ 1AT concentration ( $p < 0.001$  all comparisons). The association rate constant for neutrophil elastase of the purified S protein was less than that of the normal molecule (S-type  $7.1 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{sec}^{-1}$ ,  $M_1$ -type  $9.6 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{sec}^{-1}$ ,  $p < 0.001$ ) but much greater than that for the Z molecule ( $p < 0.001$ ). Exposure of the purified S protein to an increasing oxidant burden resulted in a dose dependent reduction in the ability of the molecule to inhibit neutrophil elastase in a fashion parallel to that of the  $M_1$  and Z proteins. Quantification of ELF  $\alpha$ 1AT levels and anti-neutrophil elastase capacity demonstrated that the SS ELF parameters were, as in plasma, intermediate between  $M_1$  homozygotes and Z homozygotes. Using the association rate constant together with the quantification of ELF  $\alpha$ 1AT levels, the "in vivo lung inhibition time" was estimated, yielding an assessment of the relative anti-neutrophil elastase screen of the PiSS lower respiratory tract: Interestingly, while the in vivo inhibition time of S molecules is longer than that of  $M_1$  homozygotes, it is not nearly as long as that of the Z individuals, consistent with the knowledge that the S



homozygous state does not confer an increased risk of emphysema to the affected individual.

Unlike the Z  $\alpha$ 1AT allele (val<sup>213</sup>-->ala, glu<sup>342</sup>-->lys) in which the serum deficiency results from intracellular aggregation of the newly synthesized  $\alpha$ 1AT with consequent decreased secretion, the basis of the deficiency associated with the S allele (glu<sup>264</sup>-->val) is unknown. One hypothesis to explain the reduced  $\alpha$ 1AT serum levels associated with the S allele is that the molecular defect causes disruption of an intramolecular salt bridge resulting in instability of the newly synthesized  $\alpha$ 1AT with consequent intracellular degradation. To evaluate this hypothesis, blood monocytes, cells that express the  $\alpha$ 1AT gene, were evaluated from individuals homozygous for M and S type  $\alpha$ 1AT. Northern analysis using a <sup>32</sup>P-labeled  $\alpha$ 1AT cDNA probe showed that both MM and SS monocytes express 1.75 kb  $\alpha$ 1AT mRNA transcripts and dot blot analysis revealed that the  $\alpha$ 1AT mRNA levels were similar ( $p>0.3$ ). To evaluate post-transcriptional processing, [<sup>35</sup>S] methionine pulse-chase studies using anti- $\alpha$ 1AT antibody immunoprecipitation showed that after a 1 hr pulse and a 4 hr chase period, both SS and MM monocytes secreted a mature form of  $\alpha$ 1AT. However, SS monocytes secreted significantly less ( $42\pm 10\%$ ,  $p<0.01$ ) mature  $\alpha$ 1AT than MM monocytes. After a 1 hr pulse period, newly synthesized precursor  $\alpha$ 1AT could be isolated from cellular lysates of both MM and SS monocytes. However, the amount of newly synthesized  $\alpha$ 1AT isolated from cellular lysates of SS monocytes was significantly less ( $34\pm 6\%$ ,  $p<0.01$ ) than the amount isolated from MM monocytes. When similar pulse chase studies were carried out in the presence of tunicamycin (5  $\mu$ g/ml), an inhibitor of core N-linked oligosaccharide side chain addition, both SS and MM monocytes secreted nonglycosylated  $\alpha$ 1AT, but the SS monocytes secreted 100-fold less ( $p<0.01$ ). Thus, while the glu<sup>264</sup>-->val S mutation is associated with normal  $\alpha$ 1AT mRNA transcription, it is associated with abnormal intracellular processing of the  $\alpha$ 1AT molecule at the post-transcriptional level, likely because the mutation renders the S-type protein less stable and thus more susceptible to intracellular proteolysis.

In most cases of  $\alpha$ 1AT deficiency associated with emphysema the serum levels of  $\alpha$ 1AT are decreased, but are present, usually in the range of 10 to 20% of that found in normals. There are cases; however, in which no  $\alpha$ 1AT can be detected at all. This form of  $\alpha$ 1AT deficiency is called the "null-null" state, i.e., neither of the two parental  $\alpha$ 1AT genes are expressed such that they produce insufficient  $\alpha$ 1AT to be detected in the serum. There have been very few cases of the "null-null" state reported. One of these, an individual homozygous for the  $\alpha$ 1AT gene null bellingham, has emphysema and is currently being treated with augmentation therapy with  $\alpha$ 1AT purified from pooled human plasma. Prior studies from our laboratory have demonstrated that this individual has  $\alpha$ 1AT genes that are grossly intact but the cells that normally express these genes contain no detectable  $\alpha$ 1AT mRNA transcripts. To define the genetic defect responsible for this null bellingham null bellingham state, we have cloned and sequenced one  $\alpha$ 1AT gene of this individual. Then, using oligonucleotide probes directed toward the observed single mutation in a coding exon that distinguishes this sequence from the normal  $\alpha$ 1AT sequence, we have evaluated the genomic DNA of the index case and the parents to demonstrate that this rare  $\alpha$ 1AT phenotype resulted from the homozygous inheritance of a stop codon in a coding exon.



The promoter region, coding exons, and all exon-intron junctions were normal except for a single base substitution in exon III, causing the normal Lys<sup>217</sup> (AAG) to become a stop codon (TAG). Evaluation of genomic DNA of family members using oligonucleotides directed towards this region demonstrated that the index case had inherited this mutation in a homozygous fashion. Although the consequences to the individual (i.e., emphysema) are identical to that associated with the common homozygous Z mutation, the homozygous null bellingham form of  $\alpha$ 1AT deficiency has a very different genetic basis.

In another study, we determined the molecular basis for a newly recognized form of a null  $\alpha$ 1AT allele, null granite falls. The  $\alpha$ 1AT gene null granite falls was identified and cloned from genomic DNA of an individual with severe  $\alpha$ 1AT deficiency and emphysema resulting from the heterozygous inheritance of the null granite falls and Z  $\alpha$ 1AT genes. Restriction endonuclease mapping of null granite falls demonstrated no gross deletions, additions, or rearrangements in 16 kb of genomic DNA centered around the 10 kb of the  $\alpha$ 1AT gene. Sequencing of the 5' flanking region, all five coding exons, and all exon-intron junctions of null granite falls demonstrated it was identical to the common normal M1(ala<sup>213</sup>)  $\alpha$ 1AT gene except for two bases; a single deletion in the condon for amino acid tyr<sup>160</sup> of the mature protein and a single base substitution 168 bp 5' to exon I. Although no role for the promoter region mutation could be assigned, the coding exon deletion [tyr(TAC)->(TA\_)] resulted in a frameshift causing a stop coding to be formed approximately 44% from the N-terminus of the precursor protein. Using oligonucleotide probes to evaluate the family of the index case demonstrated that the deletion->frameshift/stop mutation was inherited in a autosomal codominant fashion. Thus, although the molecular basis for  $\alpha$ 1AT deficiency of the  $\alpha$ 1AT null allele such as null granite falls is very different from the molecular basis of the more common deficient alleles such as Z, and the rarer allele null bellingham the phenotype consequences of these genes are similar, i.e., severe  $\alpha$ 1AT deficiency and an association of a high risk for the development of emphysema.

In addition to Z and S, the "deficient" category includes a group of relatively rare alleles associated with low serum  $\alpha$ 1AT levels and, when paired with another deficient allele, emphysema. To begin to define such alleles, we cloned both  $\alpha$ 1AT genes from a non-Z, non-S  $\alpha$ 1AT deficient (serum levels 6 mg/dl) 43-year-old former smoker with severe emphysema. Isoelectric focusing and immunoblotting of serum of the index case demonstrated that the major bands were among those of the M-family. A family study suggested the deficiency gene was inherited in a heterozygous fashion with a "null" alleles. Genomic DNA was cloned into EMBL3 and five clones were selected with an  $\alpha$ 1AT cDNA probe. The restriction endonuclease PvuII detected a polymorphism in exon II of all clones, suggesting a point mutation in this region. Sequencing of the promoter region, all five exons, and all exon-intron junctions of one of the clones demonstrated the gene sequence was identical to that of normal M1(val<sup>213</sup>) gene except for a mutation in the codon for amino acid 41 (leu<sup>41</sup>CTG->pro<sup>41</sup>CCG), a mutation that resulted in the deletion of a PvuII cutting site. Purification of this  $\alpha$ 1AT protein (referred to as "M\_procida" based on the birthplace of the index case) from the plasma of the index case revealed that it had a normal  $(7.0 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{sec}^{-1})$  association rate constant for neutrophil elastase. Interest-



ingly, analysis of the 3-dimensional crystallographic structure of  $\alpha$ 1AT revealed that residue 41 resides in an external  $\alpha$ -helix-rich domain. In this regard, the leu<sup>41</sup>->pro modification may cause derangement of the tertiary structure of the molecule, leading to instability during post-translation processing or in the extracellular milieu, resulting in the deficiency state.

In patients with  $\alpha$ 1AT deficiency, the development of emphysema is believed to be caused by the unchecked action of proteases on lung tissue. We evaluated the feasibility, safety, and biochemical efficacy of intermittent infusions of  $\alpha$ 1AT in the treatment of patients with  $\alpha$ 1AT deficiency. Twenty-one patients were given 60 mg of active plasma-derived  $\alpha$ 1AT per kilogram of body weight, once a week for up to six months. After a steady state had been reached, the group had trough serum levels of  $\alpha$ 1AT of  $126 \pm 1$  mg per deciliter as compared with  $30 \pm 1$  mg per deciliter before treatment, and serum anti-neutrophil elastase capacities of  $13.3 \pm 0.1$   $\mu$ M as compared with  $5.4 \pm 0.1$   $\mu$ M. The  $\alpha$ 1AT level in the epithelial lining fluid of the lungs was  $0.46 \pm 0.16$   $\mu$ M before treatment, and the anti-neutrophil elastase capacity was  $0.81 \pm 0.13$   $\mu$ M. Six days after infusion,  $\alpha$ 1AT levels ( $1.89 \pm 0.17$   $\mu$ M) and anti-neutrophil elastase capacities ( $1.65 \pm 0.13$   $\mu$ M) in the lining fluid were significantly increased ( $P < 0.0001$ ). Because of the chronicity of the disorder and the lack of sensitive measures of lung destruction, the clinical efficacy of this therapy could not be studied rigorously. No changes in lung function were observed in our patients over six months of treatment. The only important adverse reactions to the 507 infusions were four episodes of self-limited fever. This study demonstrates that infusions of  $\alpha$ 1AT derived from plasma are safe and can reverse the biochemical abnormalities in serum and lung fluid that characterize this disorder. Together with lifetime avoidance of cigarette smoking, replacement therapy with  $\alpha$ 1AT may be a logical approach to long-term medical treatment.

The null-null phenotype of  $\alpha$ 1AT, a phenotype characterized by no detectable  $\alpha$ 1AT in serum, presents a rare opportunity to examine the contribution of  $\alpha$ 1AT to the anti-neutrophil elastase protection of the lower respiratory tract. The subject, a 35-yr-old lifetime nonsmoker with moderate emphysema, has been characterized as having  $\alpha$ 1AT serum levels of zero resulting from the homozygous inheritance of  $\alpha$ 1AT genes that do not express detectable  $\alpha$ 1AT mRNA transcripts. Evaluation of the anti-neutrophil elastase capacity of the null-null serum showed it was <5% of normal, whereas that of the epithelial lining fluid (ELF) of the lower respiratory tract was 13% of normal. However, after 60 mg/kg of intravenously administered  $\alpha$ 1AT augmentation therapy once weekly for 4 wks, the serum  $\alpha$ 1AT levels peaked at >300 mg/dl, trough levels just prior to the next infusion were  $81 \pm 2$  mg/dl, and the average serum level integrated for the month of infusions was 138 mg/dl. Consistent with this serum rise in  $\alpha$ 1AT, the serum anti-neutrophil elastase capacity increased in parallel ( $r = 0.98$ ). Importantly, evaluation of the ELF 2 and 6 days after infusion demonstrated increases of  $\alpha$ 1AT levels (range 1.4 to 2.1  $\mu$ M) and anti-neutrophil elastase capacity (range 1.6 to 2.5  $\mu$ M), values within the lower range of normal. Furthermore, the lung ELF  $\alpha$ 1AT levels rose in direct proportion to the serum  $\alpha$ 1AT levels, and the ELF anti-neutrophil elastase capacity rose in direct proportion to the ELF  $\alpha$ 1AT levels. Taken together, these findings demonstrate that  $\alpha$ 1AT





provides >85% of the anti-neutrophil elastase protection for the lower respiratory tract and that this protection can be modulated by the blood levels of  $\alpha$ 1AT.

Typically, inactivation of serine proteases by protein inhibitors involves the formation of a very stable bimolecular complex between the active sites of the inhibitor and the enzyme. The reaction mechanism and the structure of the inhibition complex are well understood for several inhibitor classes, the most detailed analysis coming from X-ray crystallographic studies of the complexes. For the plasma protease inhibitor family, including  $\alpha$ 1AT, much less information is available. Attempts have been made to determine the 3-D structure of native  $\alpha$ 1AT but successful crystallography was achieved only with a proteolytically cleaved form which, because of a large conformational change, gave no information concerning the geometry of the active site. Nevertheless it is clear that the  $\alpha$ 1AT active site acts as a pseudosubstrate for the cognate protease and that the central P1 Met residue (position 358) plays a crucial role in the inhibition process. The importance of the P1 Met has been demonstrated by site-specific mutagenesis studies in which this residue was replaced by val recombinant  $\alpha$ 1AT (val<sup>358</sup>) produced in yeast or *Escherichia coli* retained elastase inhibitory capacity but, unlike the native form, was resistant to inactivation by chemical or biological oxidants. Thus the presence of Met sulfoxide at position 358 is sufficient to inactivate the molecule. Several studies have indicated that such oxidative inactivation occurs in the lungs of heavy cigarette smokers and therefore may be involved in the pathogenesis of acquired emphysema. Comparison of the active site sequences of the plasma protease inhibitor family, for example antithrombin III (ATIII),  $\alpha$ 1 antichymotrypsin and  $\alpha$ 1AT, shows that the residue of P1 corresponds to the specificity of the cognate protease. Moreover, direct evidence that the  $\alpha$ 1AT P1 residue is crucial in determining the specificity of inhibition was provided by the identification of a natural variant ( $\alpha$ 1AT Pittsburgh) which contains a P1 Met<sup>358</sup>  $\rightarrow$  Arg substitution. The single case described led to a fatal hemorrhagic disorder resulting from the potent thrombin inhibition capacity of the mutant inhibitor. Characterization of *E. coli*-produced recombinant  $\alpha$ 1AT (Arg<sup>358</sup>) showed that, in addition to thrombin, the P1 Met  $\rightarrow$  Arg substitution results in strong inhibition of other Arg-specific serine proteases, such as kallikrein and factor XII. We have investigated in more depth the effect of amino acid replacements at the P1 and subsite positions of the  $\alpha$ 1AT active site. Seven active site variants of human  $\alpha$ 1AT were produced in *Escherichia coli* following site-specific mutagenesis of the  $\alpha$ 1AT complementary DNA.  $\alpha$ 1AT (ala<sup>358</sup>),  $\alpha$ 1AT (ile<sup>358</sup>) and  $\alpha$ 1AT (val<sup>358</sup>) were efficient inhibitors of both neutrophil and pancreatic elastases, but not of cathepsin G.  $\alpha$ 1AT (ala<sup>358</sup>, val<sup>358</sup>) and  $\alpha$ 1AT (phe<sup>358</sup>) specifically inhibited pancreatic elastase and cathepsin G respectively. The most potent inhibitor of neutrophil elastase was  $\alpha$ 1AT (leu<sup>358</sup>), which also proved to be effective against cathepsin G. The  $\alpha$ 1AT(arg<sup>358</sup>) variant inactivated thrombin with kinetics similar to antithrombin III in the presence of heparin. Electrophoretic analysis showed that SDS-stable high molecular weight complexes were formed between the mutant inhibitors and the cognate proteases in each case. These data indicate that effective inhibition occurs when the  $\alpha$ 1AT P1 residue (position 358) corresponds to the primary specificity of the target protease. Moreover, alteration of the P3 residue (position 356) can further modify the reactivity of the inhibi-



tor. Two of the variants have therapeutic potential:  $\alpha$ 1AT (leu<sup>358</sup>) may be more useful than plasma  $\alpha$ 1AT in the treatment of destructive lung disorders and  $\alpha$ 1AT (arg<sup>358</sup>) could be effective in the control of thrombosis.

The current concept relating to the pathogenesis of emphysema associated with cigarette smoking is that an imbalance exists within the lower respiratory tract between neutrophil elastase and the local anti-neutrophil elastase screen, enabling uninhibited neutrophil elastase to destroy the alveolar structures over time. The possible role of the alveolar macrophage in contributing to this imbalance was investigated by evaluating the ability of cigarette smokers' alveolar macrophages to inactivate  $\alpha$ 1AT, the major anti-neutrophil elastase of the human lower respiratory tract. In vitro, alveolar macrophages of smokers spontaneously released 2.5-fold more superoxide anion and 8-fold more  $H_2O_2$  than macrophages of nonsmokers ( $p < 0.01$ , both comparisons). Using a model system which reproduced the relative amounts of alveolar macrophages and  $\alpha$ 1AT found in the epithelial lining fluid of the lower respiratory tract, we observed that smokers' macrophages caused a  $60 \pm 5\%$  reduction in the ability of  $\alpha$ 1AT to inhibit neutrophil elastase. In marked contrast, under the same conditions, non-smokers' macrophages had no effect upon the anti-neutrophil elastase function of  $\alpha$ 1AT. Addition of superoxide dismutase and catalase prevented inactivation of  $\alpha$ 1AT by smokers' macrophages, implying that the release of oxidants mediated the inactivation of  $\alpha$ 1AT. In addition, by utilizing a recombinant DNA produced modified form of  $\alpha$ 1AT containing an active site substitution (met<sup>358</sup>  $\rightarrow$  val), the inactivation of  $\alpha$ 1AT by smokers' alveolar macrophages was prevented, suggesting that the smokers' macrophages inactivate the  $\alpha$ 1AT by oxidizing the active site of the  $\alpha$ 1AT molecule. These results suggest that in cigarette smokers, the alveolar macrophage can modulate the activity of  $\alpha$ 1AT as an inhibitor of neutrophil elastase and thus play a role in the pathogenesis of emphysema associated with cigarette smoking.

To evaluate the potential use of recombinant DNA produced  $\alpha$ 1AT to augment the lung anti-neutrophil elastase defenses in  $\alpha$ 1AT deficiency, we compared the kinetics of intravenously administered recombinant produced  $\alpha$ 1-antitrypsin (r $\alpha$ 1AT) and purified normal human plasma  $\alpha$ 1-antitrypsin (p $\alpha$ 1AT) in the blood and lung of rhesus monkeys. The r $\alpha$ 1AT was produced in yeast transformed with an expressing plasmid containing a full length human  $\alpha$ 1-antitrypsin cDNA and purified to  $>99\%$  homogeneity. The r $\alpha$ 1AT has a MW of 45,000 daltons, no carbohydrates and is identical in sequence to normal plasma  $\alpha$ 1AT except for an additional N-terminal acetyl-methionine. Despite its lack of carbohydrates, the r $\alpha$ 1AT inhibited human neutrophil elastase with an association rate constant similar to that of p $\alpha$ 1AT. Rhesus monkeys were infused intravenously with 120 mg/kg of r $\alpha$ 1AT ( $n=13$ ) or p $\alpha$ 1AT ( $n=12$ ) and the serum, urine, and lung epithelial lining fluid (ELF) concentrations of these molecules quantified at various intervals. Although the initial serum levels of the r $\alpha$ 1AT and p $\alpha$ 1AT were both dose dependent, the p $\alpha$ 1AT remained in the blood for at least 4 days while the r $\alpha$ 1AT disappeared rapidly, such that it was barely detectable at 24 hr and undetectable thereafter. Strikingly, while no p $\alpha$ 1AT was detectable in the urine at any time, 38% of the intravenously administered r $\alpha$ 1AT was excreted within 3 hr. Similar to its behavior in man, the p $\alpha$ 1AT diffused into the lung such that its concentration in the ELF of the lower respiratory tract



1 to 4 days after infusion was approximately 10% that in serum. Interestingly, the r $\alpha$ 1AT molecule also diffused into the lung, with ELF levels at 24 hr similar to that of the p $\alpha$ 1AT. Furthermore, while the r $\alpha$ 1AT ELF levels declined by 48 hr and 96 hr to below those of the p $\alpha$ 1AT ELF levels, the r $\alpha$ 1AT ELF levels exceeded those in blood at the same time points. Importantly, like the p $\alpha$ 1AT, intravenous administration of r $\alpha$ 1AT resulted in a significant augmentation of the anti-neutrophil elastase capacity of the ELF. Thus, in primates, human based r $\alpha$ 1AT has very different pharmacokinetics than does human p $\alpha$ 1AT, likely because of its modified charge and/or conformation. Despite this, however, the r $\alpha$ 1AT does diffuse into the lung and augments the anti-neutrophil elastase capacity of the epithelial lining fluid of the lower respiratory tract, suggesting it has potential as a therapeutic agent in the treatment of disorders such as  $\alpha$ 1AT deficiency.

Recombinant DNA directed human  $\alpha$ 1-antitrypsin (r $\alpha$ 1AT) functions identically to normal human  $\alpha$ 1AT as an elastase inhibitor but its lack of carbohydrate side chains and consequent short half-life following intravenous administration precludes its use in treating  $\alpha$ 1AT deficiency by intermittent intravenous infusion. Aerosol administration would target the r $\alpha$ 1AT appropriately and thus circumvents this problem, but it would be efficacious only if the r $\alpha$ 1AT reaches the alveoli and diffuses across the alveolar walls. To evaluate this approach, human  $\alpha$ 1AT cDNA-directed yeast-produced r $\alpha$ 1AT was administered intravenously or by aerosol to sheep (n=11) and the concentrations of r $\alpha$ 1AT over time measured in plasma, lung epithelial lining fluid (ELF), and lung lymph. Using a dose of 60 mg/kg, intravenous infusion resulted in lung ELF levels of  $400 \pm 100$  nM after 2 hrs. In contrast, using a nebulization system that generated >95% of particles  $\leq 5 \mu\text{m}$  and  $34 \pm 2\%$   $\leq 2 \mu\text{m}$ , a dose of only 2.5 mg/kg of aerosolized r $\alpha$ 1AT resulted in the same ELF levels at 2 hrs ( $p > 0.1$ ). Importantly, the aerosolized r $\alpha$ 1AT appeared in lung lymph in a time dependent manner (1 hr  $2 \pm 1$  nM, 2 hr  $13 \pm 6$  nM, 3 hr  $27 \pm 17$  nM, 4 hr  $117 \pm 30$  nM), driven by a concentration gradient in ELF (2 hr  $400 \pm 50$  nM). Thus, r $\alpha$ 1AT can be aerosolized into sufficient size to access the alveolar spaces and in vivo reaches the epithelial surface and interstitium of the lower respiratory tract where it could augment the anti-elastase defenses, thus potentially useful for therapy of  $\alpha$ 1AT deficiency.

The concept of treatment is similar for all forms of  $\alpha$ 1AT deficiency: enhance the anti-neutrophil elastase protection in the lower respiratory tract. The current approach has been to augment levels by the infusion of  $\alpha$ 1AT purified from pooled human plasma. Alternatively, it should be possible to utilize a human  $\alpha$ 1AT cDNA to direct the production of  $\alpha$ 1AT. The normal human  $\alpha$ 1AT cDNA has been used to transform *Escherichia coli* and yeast, but the recombinant  $\alpha$ 1AT molecules produced lack carbohydrates; consequently, their plasma half-life is markedly reduced, obviating the intravenous route for their administration. As an approach to augment  $\alpha$ 1AT levels in this disorder with physiologically normal human  $\alpha$ 1AT, we have integrated a full-length normal human  $\alpha$ 1AT cDNA into the genome of mouse fibroblasts. To accomplish this, the retroviral vector N2 was modified by inserting the simian virus 40 early promoter followed by the  $\alpha$ 1AT cDNA. Southern analysis demonstrated that the intact cDNA was present in the genome of selected clones of the transfected murine fibroblasts  $\psi$ 2 and



infected NIH/3T3. The clones produced three mRNA transcripts (5.8, 4.8, and 2.4 kilobases) containing human  $\alpha$ 1AT sequences, secreted on  $\alpha$ 1AT molecule recognized by an anti-human  $\alpha$ 1AT antibody, with the same molecular mass (52 kDa) as normal human  $\alpha$ 1AT and that complexed with and inhibited human neutrophil elastase. The  $\psi$ 2 produced  $\alpha$ 1AT was glycosylated, and when infused intravenously into mice, it had a serum half-life similar to normal  $\alpha$ 1AT purified from human plasma and markedly longer than that of nonglycosylated human  $\alpha$ 1AT cDNA-directed yeast-produced  $\alpha$ 1AT. These studies demonstrate the feasibility of using a retroviral vector to insert the normal human  $\alpha$ 1AT cDNA into non- $\alpha$ 1AT-producing cells, resulting in the synthesis and secretion of physiologically "normal" human  $\alpha$ 1AT.

As a model approach to "gene therapy" of  $\alpha$ 1AT deficiency, a retroviral vector was used to insert the human  $\alpha$ 1AT cDNA into the genome of mouse fibroblasts to create a clonal population of mouse fibroblasts secreting human  $\alpha$ 1AT. After demonstrating that this clone of fibroblasts produced  $\alpha$ 1AT after more than 100 population doublings in the absence of selection pressure, the clone was transplanted into the peritoneal cavities of nude mice. When the animals were evaluated 4 weeks later, human  $\alpha$ 1AT was detected in both sera and the epithelial surface of the lungs. The transplanted clone of fibroblasts could be recovered from the peritoneal cavities of those mice and demonstrated to still be producing human  $\alpha$ 1AT. Thus, even after removal of selective pressure, a single clone of retroviral vector-infected cells that expressed an exogenous gene in vitro, continued to do so in vivo, and when recovered, continued to produce the product of the exogenous gene.

$\alpha$ 1AT, the major inhibitor of neutrophil elastase (NE) in the human lower respiratory tract (LRT), interacts with NE through the  $\alpha$ 1AT met<sup>358</sup>-ser<sup>359</sup> active site with an association rate constant ( $K_{\text{assoc}}$ ) of  $10^7 \text{ M}^{-1}\text{sec}^{-1}$ . To evaluate the hypothesis that  $\alpha$ 1AT in the LRT of cigarette smokers is less capable of inhibiting NE in a time-dependent fashion than LRT  $\alpha$ 1AT of nonsmokers, the  $K_{\text{assoc}}$  of LRT  $\alpha$ 1AT for NE was compared in current smokers ( $n=7$ ,  $15 \pm 4$  pack-yr, normal lung function) and nonsmokers ( $n=12$ ), normal lung function). To accomplish this,  $\alpha$ 1AT was purified ( $>95\%$ ) from lavage fluid using affinity and molecular sieve chromatography and the  $K_{\text{assoc}}$  for NE quantified using a time-dependent (0-7) min assay with equal amounts of  $\alpha$ 1AT and NE. The  $K_{\text{assoc}}$  of smoker plasma  $\alpha$ 1AT ( $9.5 \pm 0.5 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ ) was similar to that of nonsmoker plasma  $\alpha$ 1AT ( $9.3 \pm 0.7 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ ,  $p > 0.5$ ). In marked contrast, the  $K_{\text{assoc}}$  of smoker LRT  $\alpha$ 1AT ( $6.5 \pm 0.4 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ ) was significantly lower ( $p < 0.01$ ) than that of nonsmoker LRT  $\alpha$ 1AT ( $8.1 \pm 0.5 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ ). Furthermore, the smoker LRT  $\alpha$ 1AT  $K_{\text{assoc}}$  was significantly less ( $p < 0.01$ ) than that of autologous plasma. But in the context of the concentration of  $\alpha$ 1AT in the LRT epithelial lining fluid, the inhibition time for NE of smoker LRT  $\alpha$ 1AT was 2-fold less than that of nonsmoker LRT  $\alpha$ 1AT (smoker:  $0.35 \pm 0.05$  sec vs nonsmoker:  $0.17 \pm 0.05$  sec  $p < 0.01$ ), i.e., for the concentration of  $\alpha$ 1AT in the LRT, it takes twice as long for an equivalent burden of NE to be inhibited in the smoker's lung compared to the non-smoker's lung. Thus, despite its presence in similar amounts, when evaluated in a kinetic fashion against its true target protease,  $\alpha$ 1AT in the lung of smokers was less able to provide a competent anti-neutrophil elastase screen than the  $\alpha$ 1AT of nonsmokers. These observations support the concept that cigarette smoking is associated with a shift in the balance of





anti-neutrophil elastase-neutrophil elastase in the lung toward the elastase, making the lung more vulnerable to elastase and hence to destruction.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

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## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Destructive Lung Disease

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Pulmonary Branch

## SECTION

## INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

12.7

## PROFESSIONAL:

8.7

## OTHER:

4

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

There are 2 million individuals in the U.S.A. with emphysema. Two percent develop the disease because of inheritance of a deficiency of alpha 1-anti-trypsin (AAT), an antiprotease that protects the lower respiratory tract from destruction mediated by elastase released by neutrophils. Cloning, sequencing and oligonucleotides have been used to detect specific mutations in AAT gene. The "null" AAT state is associated with an intact gene but no detectable AAT mRNA. Alveolar macrophages produce AAT, thus providing the protein at the site of disease. Site directed mutagenesis has been used to produce a recombinant AAT molecule in E.coli that is oxidation resistant. Therapy of AAT deficiency with AAT purified from pooled plasma has demonstrated that the anti-neutrophil-elastase defenses of the lung can be re-established with intermittent intravenous administration of 60 mg/kg AAT.

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Other Investigators: (continued)

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Alpha 1-antitrypsin ( $\alpha$ 1AT), a 52,000 dalton serum glycoprotein produced by hepatocytes and mononuclear phagocytes, functions to inhibit neutrophil elastase, a proteolytic enzyme capable of destroying all protein components of connective tissue. The  $\alpha$ 1AT gene is highly pleomorphic; more than 30 different alleles have been described. The  $\alpha$ 1AT phenotype, referred to as the Pi (protease inhibitor) type, represents the codominant expression of the two parental  $\alpha$ 1AT alleles. The most common  $\alpha$ 1AT alleles in the U.S.A. are those of the M-family (combined frequency greater than 0.90), the S type (frequency 0.02-0.04) and the Z type (0.01-0.02). The clinical interest in these  $\alpha$ 1AT alleles is based on the knowledge that inheritance of the phenotypes PiZZ and PiSZ are associated with an increased risk for the development of emphysema (in adults) and/or liver disease (in children).

In the context that Pulmonary Branch, NHLBI, is a national referral center for adults in the USA with symptomatic emphysema with  $\alpha$ 1AT deficiency, we evaluated 124 such individuals using a variety of routine clinical parameters in order to draw conclusions concerning the characteristics of such a population. The typical adult  $\alpha$ 1AT deficient patient with symptomatic emphysema has an  $\alpha$ 1AT level of 30 mg/dl, and almost always has the PiZZ phenotype. This individual was most often male, Caucasian, an ex-smoker and became dyspneic at ages 25 to 40. Most routine blood tests were normal. The chest X-ray and ventilation-perfusion studies typically demonstrated abnormalities with a lower zone distribution and about one-third had evidence suggestive of pulmonary hypertension. Lung function tests were typical for emphysema. The FEV<sub>1</sub> and DLCO were the parameters most dramatically reduced, and the annual rate of decline of those parameters was greater than that of the general population. The cumulative probability of survival of PiZZ deficient adults with pulmonary symptoms indicated a significantly shortened lifespan with a mean survival of 16% at age 60 compared with 85% for normal individuals.

The emphysema of  $\alpha$ 1AT deficiency is conceptualized to result from insufficient  $\alpha$ 1AT allowing neutrophil elastase (NE) to work unimpeded to destroy lung parenchyma. Lavage studies have shown that  $\alpha$ 1AT deficient individuals have increased numbers of neutrophils (PMN) in their lungs compared to normal individuals. With the knowledge that alveolar macrophages (AM) have surface receptors for NE, we hypothesized that PMN accumulation in lung parenchyma in  $\alpha$ 1AT deficiency may result from release of PMN chemotactic activity by AM as they bind and internalize uninhibited NE. To evaluate this hypothesis, we compared spontaneous PMN chemotactic activity release by AM from  $\alpha$ 1AT deficient individuals to that by AM of normals using a conventional chemotactic assay with human PMN as the responding cells.  $\alpha$ 1AT deficient AM spontaneously released more than 3-fold as much PMN chemotactic activity as normal AM ( $\alpha$ 1AT deficient  $305 \pm 63$  U/ $10^6$  AM-3 hr vs normal  $82 \pm 94$ ,  $p < .01$ ). Consistent with this hypothesis, the incubation of normal AM with NE resulted in an NE dose dependent increase in PMN chemotactic activity release (100nM NE,  $37^\circ$ :  $384 \pm 90$  U/ $10^6$  AM-3 hr,  $p < .01$  vs non-NE exposed AM), similar to the level seen following incubation of normal AM with zymosan ( $439 \pm 92$  U/ $10^6$  AM-3 hr). However, preincubation of NE with  $\alpha$ 1AT prior to its addition to normal AM led to an  $\alpha$ 1AT dose-dependent decrease in release of PMN chemotactic activity. HPLC and molecular sieve chromatography demonstrated that the PMN chemotactic signal(s) released by  $\alpha$ 1AT deficient AM and NE stimulated normal AM included





leukotriene B<sub>4</sub> (LTB<sub>4</sub>). These results suggest that a mechanism for the increased PMN in the lungs of  $\alpha$ 1AT deficient individuals may be the release of LTB<sub>4</sub> by AM as they bind free elastase. In the  $\alpha$ 1AT deficient lung this may establish a cycle in which non- $\alpha$ 1AT bound NE is taken up by AM, which then release LTB<sub>4</sub>, causing the recruitment of still more PMN, with their elastase. Thus, a chronically increased PMN number and NE burden is maintained, leading to accelerated lung destruction in  $\alpha$ 1AT deficiency.

The entire nucleotide sequence of the protein coding region sequence of the  $\alpha$ 1AT Z gene was determined using conventional cloning methods. In addition to the glu<sup>342</sup> to lys<sup>342</sup> mutation in exon V which has been previously identified by peptide analysis, another point mutation (GTG to GCG in exon III) in the gene sequence predicts a second amino acid substitution (val<sup>213</sup> to ala<sup>213</sup>) in the Z protein. This val<sup>213</sup> to ala<sup>213</sup> mutation was confirmed to be a general finding in Z type  $\alpha$ 1AT gene by evaluating genomic DNA from 40 Z alleles using synthetic oligonucleotide gene probes directed toward the mutated exon III sequences in the Z gene. Furthermore, the exon III val<sup>213</sup> to ala<sup>213</sup> mutation eliminates a BstEII restriction endonuclease site in the  $\alpha$ 1AT Z gene, allowing rapid identification of this val<sup>213</sup> to ala<sup>213</sup> substitution at the genomic DNA level. Surprisingly, when genomic DNA samples from individuals thought to be homozygous for the M1 gene (the most common  $\alpha$ 1AT normal allele) were evaluated with BstEII, 23% of the M1 alleles were BstEII site negative, thus identifying a new form of M1 (i.e., M1(ala<sup>213</sup>)), likely identical to M1 but with an isoelectric focusing "silent" amino acid substitution (val<sup>213</sup> to ala<sup>213</sup>). Although the relative importance of the newly identified exon III val<sup>213</sup> to ala<sup>213</sup> mutation to the pathogenesis of the abnormalities associated with the Z gene is not known, it is likely that M1(Ala<sup>213</sup>) gene represents a common "normal" polymorphism of the  $\alpha$ 1AT gene that served as an evolutionary intermediate between the M1(Val<sup>213</sup>) and Z genes.

In normal individuals with the homozygous inheritance of the common M1-type  $\alpha$ 1AT gene, the serum  $\alpha$ 1AT levels are 150 to 350 mg/dl. In contrast, in the common form of  $\alpha$ 1AT deficiency associated with homozygous inheritance of the Z-type  $\alpha$ 1AT gene, serum  $\alpha$ 1AT levels are invariably less than 50 mg/dl. In this context, it is accepted that the reduction of  $\alpha$ 1AT levels to <50 mg/dl is sufficient to permit the burden of neutrophils in the lower respiratory tract to slowly destroy the lung parenchyma. However, while this logic gives a compelling basis for understanding the pathogenesis of the emphysema associated with  $\alpha$ 1AT deficiency, it ignores the data from epidemiologic studies that has convincingly shown that while serum  $\alpha$ 1AT levels of 150 mg/dl are the lower limit of normal individuals, individuals with levels of  $\geq$ 80 mg/dl are at no increased risk for the development of emphysema beyond the risk for the general population. Thus, while individuals with homozygous inheritance of the Z gene commonly have  $\alpha$ 1AT levels in the 25 to 45 mg/dl range, those with only 2 to 2.5-fold more  $\alpha$ 1AT have no increased risk for emphysema, i.e., the epidemiologic data leads to the conclusion that a relatively small reduction in  $\alpha$ 1AT levels is sufficient to place the individual at high risk for the development of emphysema. While such a reduction in the anti-neutrophil elastase screen for the lower respiratory tract could be sufficient to cause such a high risk for disease, we have hypothesized that the mutations in the coding sequence for the  $\alpha$ 1AT gene may result, in addition to a reduction in



the serum levels of  $\alpha$ 1AT, in a diminished ability of the  $\alpha$ 1AT molecule to work effectively as an inhibitor of neutrophil elastase. In this context, a study was designed to evaluate the concept that, in addition to the Z-type  $\alpha$ 1AT protein being present in reduced amounts, the molecule itself is less able to inhibit neutrophil elastase compared to the normal M1 molecule. To evaluate this hypothesis, the functional status of  $\alpha$ 1AT from PiZZ individuals was compared with that of  $\alpha$ 1AT from PiM1M1 individuals for its ability to inhibit neutrophil elastase (% inhibition) as well as its association rate constant for neutrophil elastase ( $K$  association). Plasma  $\alpha$ 1AT concentration, measured by radial immunodiffusion, was  $34 \pm 1$  mg/dl in PiZZ patients vs  $237 \pm 14$  mg/dl for PiM1M1 plasma, a 7-fold difference. When titrated against neutrophil elastase, the percent inhibition of PiZZ plasma was significantly less than Pi M1M1 plasma (ZZ  $78 \pm 1\%$  vs M1M1  $95 \pm 1\%$ ,  $p < 0.001$ ) as was purified Z type  $\alpha$ 1AT (ZZ,  $63 \pm 2\%$  vs M1M1  $86 \pm 2\%$ ,  $p < 0.001$ ). SDS gel comparisons of the complexes formed with M1-type  $\alpha$ 1AT and Z-type  $\alpha$ 1AT with elastase demonstrated that the Z-type  $\alpha$ 1AT elastase complexes were less stable than the M1-type  $\alpha$ 1AT-elastase complexes, thus releasing some of the enzyme to continue to function as a protease. Consistent with these observations, the  $K$  association of purified Z-type  $\alpha$ 1AT for neutrophil elastase was lower than that of M1-type  $\alpha$ 1AT (ZZ  $4.5 \pm 0.3 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$  vs M1M1  $9.7 \pm 0.4 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ ,  $p < 0.001$ ), suggesting that for the population of  $\alpha$ 1AT molecules, the active Z-type molecules take more than twice as long as the active M1-type  $\alpha$ 1AT to inhibit neutrophil elastase. Consequently, not only is there less  $\alpha$ 1AT in PiZZ individuals, but the population of Z-type  $\alpha$ 1AT molecules are less competent as an inhibitors of neutrophil elastase than M1-type  $\alpha$ 1AT molecules. This combination of defects suggests that PiZZ individuals have far less functional anti-elastase protection than suggested by the reduced concentrations of  $\alpha$ 1AT alone, further explaining their profound risk for development of emphysema.

In the course of studying the  $\alpha$ 1AT type Z gene, we identified a restriction endonuclease BstEII polymorphism in the M1 gene that predicted the existence of a previously unidentified, but relatively common allele of M, referred to as M1(ala<sup>213</sup>). We cloned both  $\alpha$ 1AT genes from an individual heterozygous for the M1(ala<sup>213</sup>) and M1(val<sup>213</sup>) alleles. Sequencing of the coding exons of both demonstrated that they are identical except for the ala-val difference at residue 213. The codominant transmission of the M1(ala<sup>213</sup>) gene was demonstrated in a family study. Evaluation of 39 genomic samples of Caucasians with the IEF allele M1 demonstrated allelic frequencies 68% for M1(val<sup>213</sup>) and 32% for M1(ala<sup>213</sup>).  $\alpha$ 1AT serum levels of individuals inheriting the M1 (ala<sup>213</sup>) gene in a homozygous fashion were in the same range as those for homozygous M1(val<sup>213</sup>) as was the rate of association of the M1(ala<sup>213</sup>) protein with neutrophil elastase. Interestingly, comparison of the M1(ala<sup>213</sup>) gene sequence to all of the known  $\alpha$ 1AT sequences at the gene and protein levels demonstrated that M1(ala<sup>213</sup>) is the closest to the baboon  $\alpha$ 1AT coding exons, suggesting that M1(ala<sup>213</sup>) is the "oldest" type human  $\alpha$ 1AT known.

Sequencing of the Z  $\alpha$ 1AT gene has demonstrated it differs from the most common normal form of  $\alpha$ 1AT [M1(val<sup>213</sup>)] in two residues, val<sup>213</sup>-> ala and glu<sup>342</sup>->lys. Since the 342 residue participates in a salt bridge (glu<sup>342</sup>, lys<sup>290</sup>) in the 3-dimensional structure of the protein, it has been suggested that the 342 mutation causes a disruption of the salt bridge result-



ing in the intracellular accumulation and reduced secretion of the Z protein by the  $\alpha$ 1AT producing cells. To directly prove this concept we modified the M<sub>1</sub>(val<sup>213</sup>) full length  $\alpha$ 1AT human cDNA by oligonucleotide directed mutagenesis utilizing the single strand DNA bacteriophage M13 and a 19 base oligonucleotide to change the sequence to M1(val<sup>213</sup>, glu<sup>342</sup>-lys) (i.e., the glu<sup>342</sup>-lys mutation on the M1(val<sup>213</sup>) background). In parallel with the normal M1(val<sup>213</sup>) cDNA, this cDNA was placed in the expression vector pSVL, which allows for gene expression in mammalian cells. Using calcium phosphate DNA transfection, log phase Cos I cells (SV40 virus transformed green monkey kidney cells) were transfected with equal amounts of DNA of either the mutated or normal  $\alpha$ 1AT sequence. RNA harvested at 72 hr and analyzed using a <sup>32</sup>P-labeled  $\alpha$ 1AT cDNA probe demonstrated the M1(val<sup>213</sup>) and M1(val<sup>213</sup>, glu<sup>342</sup>-lys) transfected cultures produced similar amounts of  $\alpha$ 1AT mRNA. However, when the transfected cells were labeled with [<sup>35</sup>S]methionine in a pulse-chase fashion and the secreted  $\alpha$ 1AT was analyzed by immunoprecipitation with anti- $\alpha$ 1-antitrypsin antibody, SDS PAGE and autoradiography, the amount of  $\alpha$ 1AT secreted by M1(val<sup>213</sup>, glu<sup>342</sup>-lys) transfected cultures was 7 $\pm$ 1 fold less ( $p < 0.01$ ) than that of the M1(val<sup>213</sup>) transfected cultures. Thus, even on a M1(val<sup>213</sup>) background a single base alteration of G-A at codon 342 clearly causes a marked reduction in the amount of secreted  $\alpha$ 1AT similar to that seen in Z homozygotes.

S-type  $\alpha$ 1-antitrypsin ( $\alpha$ 1AT) is a deficiency allele that differs from the common normal M1(val<sup>213</sup>)  $\alpha$ 1AT allele by a single amino acid (glu<sup>264</sup> to val<sup>264</sup>). To evaluate the adequacy of the anti-neutrophil elastase protection associated with the S homozygous state,  $\alpha$ 1AT plasma and lung epithelial lining fluid (ELF) levels and anti-neutrophil elastase function were analyzed in PiSS individuals. The plasma  $\alpha$ 1AT levels of SS individuals were intermediate between that of M1M1 and ZZ individuals ( $p < 0.001$  all comparisons) and the plasma neutrophil elastase inhibitory capacity paralleled the differences in  $\alpha$ 1AT concentration ( $p < 0.001$  all comparisons). The association rate constant for neutrophil elastase of the purified S protein was less than that of the normal molecule (S-type  $7.1 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ , M1-type  $9.6 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ ,  $p < 0.001$ ) but much greater than that for the Z molecule ( $p < 0.001$ ). Exposure of the purified S protein to an increasing oxidant burden resulted in a dose dependent reduction in the ability of the molecule to inhibit neutrophil elastase in a fashion parallel to that of the M1 and Z proteins. Quantification of ELF  $\alpha$ 1AT levels and anti-neutrophil elastase capacity demonstrated that the SS ELF parameters were, as in plasma, intermediate between M1 homozygotes and Z homozygotes. Using the association rate constant together with the quantification of ELF  $\alpha$ 1AT levels, the "in vivo lung inhibition time" was estimated, yielding an assessment of the relative anti-neutrophil elastase screen of the PiSS lower respiratory tract. Interestingly, while the in vivo inhibition time of S molecules is longer than that of M1 homozygotes, it is not nearly as long as that of the Z individuals, consistent with the knowledge that the S homozygous state does not confer an increased risk of emphysema to the affected individual.

Unlike the Z  $\alpha$ 1AT allele (val<sup>213</sup>  $\rightarrow$  ala, glu<sup>342</sup>  $\rightarrow$  lys) in which the serum deficiency results from intracellular aggregation of the newly synthesized  $\alpha$ 1AT with consequent decreased secretion, the basis of the deficiency associated with the S allele (glu<sup>264</sup>  $\rightarrow$  val) is unknown. One hypothesis to



explain the reduced  $\alpha$ 1AT serum levels associated with the S allele is that the molecular defect causes disruption of an intramolecular salt bridge resulting in instability of the newly synthesized  $\alpha$ 1AT with consequent intracellular degradation. To evaluate this hypothesis, blood monocytes, cells that express the  $\alpha$ 1AT gene, were evaluated from individuals homozygous for M and S type  $\alpha$ 1AT. Northern analysis using a  $^{32}\text{P}$ -labeled  $\alpha$ 1AT cDNA probe showed that both MM and SS monocytes express 1.75 kb  $\alpha$ 1AT mRNA transcripts and dot blot analysis revealed that the  $\alpha$ 1AT mRNA levels were similar ( $p>0.3$ ). To evaluate post-transcriptional processing, [ $^{35}\text{S}$ ] methionine pulse-chase studies using anti- $\alpha$ 1AT antibody immunoprecipitation showed that after a 1 hr pulse and a 4 hr chase period, both SS and MM monocytes secreted a mature form of  $\alpha$ 1AT. However, SS monocytes secreted significantly less ( $42\pm 10\%$ ,  $p<0.01$ ) mature  $\alpha$ 1AT than MM monocytes. After a 1 hr pulse period, newly synthesized precursor  $\alpha$ 1AT could be isolated from cellular lysates of both MM and SS monocytes. However, the amount of newly synthesized  $\alpha$ 1AT isolated from cellular lysates of SS monocytes was significantly less ( $34\pm 6\%$ ,  $p<0.01$ ) than the amount isolated from MM monocytes. When similar pulse chase studies were carried out in the presence of tunicamycin ( $5\text{ }\mu\text{g/ml}$ ), an inhibitor of core N-linked oligosaccharide side chain addition, both SS and MM monocytes secreted nonglycosylated  $\alpha$ 1AT, but the SS monocytes secreted 100-fold less ( $p<0.01$ ). Thus, while the  $\text{glu}^{264}\rightarrow\text{val}$  S mutation is associated with normal  $\alpha$ 1AT mRNA transcription, it is associated with abnormal intracellular processing of the  $\alpha$ 1AT molecule at the post-transcriptional level, likely because the mutation renders the S-type protein less stable and thus more susceptible to intracellular proteolysis.

In most cases of  $\alpha$ 1AT deficiency associated with emphysema the serum levels of  $\alpha$ 1AT are decreased, but are present, usually in the range of 10 to 20% of that found in normals. There are cases; however, in which no  $\alpha$ 1AT can be detected at all. This form of  $\alpha$ 1AT deficiency is called the "null-null" state, i.e., neither of the two parental  $\alpha$ 1AT genes are expressed such that they produce insufficient  $\alpha$ 1AT to be detected in the serum. There have been very few cases of the "null-null" state reported. One of these, an individual homozygous for the  $\alpha$ 1AT gene null bellingham, has emphysema and is currently being treated with augmentation therapy with  $\alpha$ 1AT purified from pooled human plasma. Prior studies from our laboratory have demonstrated that this individual has  $\alpha$ 1AT genes that are grossly intact but the cells that normally express these genes contain no detectable  $\alpha$ 1AT mRNA transcripts. To define the genetic defect responsible for this null bellingham null bellingham state, we have cloned and sequenced one  $\alpha$ 1AT gene of this individual. Then, using oligonucleotide probes directed toward the observed single mutation in a coding exon that distinguishes this sequence from the normal  $\alpha$ 1AT sequence, we have evaluated the genomic DNA of the index case and the parents to demonstrate that this rare  $\alpha$ 1AT phenotype resulted from the homozygous inheritance of a stop codon in a coding exon. The promoter region, coding exons, and all exon-intron junctions were normal except for a single base substitution in exon III, causing the normal  $\text{Lys}^{217}$  (AAG) to become a stop codon (TAG). Evaluation of genomic DNA of family members using oligonucleotides directed towards this region demonstrated that the index case had inherited this mutation in a homozygous fashion. Although the consequences to the individual (i.e., emphysema) are identical to that associated with the common homozygous Z





mutation, the homozygous null bellingham form of  $\alpha$ 1AT deficiency has a very different genetic basis.

In another study, we determined the molecular basis for a newly recognized form of a null  $\alpha$ 1AT allele, null granite falls. The  $\alpha$ 1AT gene null granite falls was identified and cloned from genomic DNA of an individual with severe  $\alpha$ 1AT deficiency and emphysema resulting from the heterozygous inheritance of the null granite falls and Z  $\alpha$ 1AT genes. Restriction endonuclease mapping of null granite falls demonstrated no gross deletions, additions, or rearrangements in 16 kb of genomic DNA centered around the 10 kb of the  $\alpha$ 1AT gene. Sequencing of the 5' flanking region, all five coding exons, and all exon-intron junctions of null granite falls demonstrated it was identical to the common normal M1(ala<sup>213</sup>)  $\alpha$ 1AT gene except for two bases; a single deletion in the codon for amino acid tyr<sup>160</sup> of the mature protein and a single base substitution 168 bp 5' to exon I. Although no role for the promoter region mutation could be assigned, the coding exon deletion [tyr(TAC)->(TA\_)] resulted in a frameshift causing a stop coding to be formed approximately 44% from the N-terminus of the precursor protein. Using oligonucleotide probes to evaluate the family of the index case demonstrated that the deletion->frameshift/stop mutation was inherited in an autosomal codominant fashion. Thus, although the molecular basis for  $\alpha$ 1AT deficiency of the  $\alpha$ 1AT null allele such as null granite falls is very different from the molecular basis of the more common deficient alleles such as Z, and the rarer allele null bellingham the phenotype consequences of these genes are similar, i.e., severe  $\alpha$ 1AT deficiency and an association of a high risk for the development of emphysema.

In addition to Z and S, the "deficient" category includes a group of relatively rare alleles associated with low serum  $\alpha$ 1AT levels and, when paired with another deficient allele, emphysema. To begin to define such alleles, we cloned both  $\alpha$ 1AT genes from a non-Z, non-S  $\alpha$ 1AT deficient (serum levels 6 mg/dl) 43-year-old former smoker with severe emphysema. Isoelectric focusing and immunoblotting of serum of the index case demonstrated that the major bands were among those of the M-family. A family study suggested the deficiency gene was inherited in a heterozygous fashion with a "null" alleles. Genomic DNA was cloned into EMBL3 and five clones were selected with an  $\alpha$ 1AT cDNA probe. The restriction endonuclease PvuII detected a polymorphism in exon II of all clones, suggesting a point mutation in this region. Sequencing of the promoter region, all five exons, and all exon-intron junctions of one of the clones demonstrated the gene sequence was identical to that of normal M1(val<sup>213</sup>) gene except for a mutation in the codon for amino acid 41 (leu<sup>41</sup>CTG->pro<sup>41</sup>CCG), a mutation that resulted in the deletion of a PvuII cutting site. Purification of this  $\alpha$ 1AT protein (referred to as "M procida" based on the birthplace of the index case) from the plasma of the index case revealed that it had a normal ( $7.0 \pm 0.1 \times 10^6$  M<sup>-1</sup>sec<sup>-1</sup>) association rate constant for neutrophil elastase. Interestingly, analysis of the 3-dimensional crystallographic structure of  $\alpha$ 1AT revealed that residue 41 resides in an external  $\alpha$ -helix-rich domain. In this regard, the leu<sup>41</sup>->pro modification may cause derangement of the tertiary structure of the molecule, leading to instability during post-translation processing or in the extracellular milieu, resulting in the deficiency state.



In patients with  $\alpha$ 1AT deficiency, the development of emphysema is believed to be caused by the unchecked action of proteases on lung tissue. We evaluated the feasibility, safety, and biochemical efficacy of intermittent infusions of  $\alpha$ 1AT in the treatment of patients with  $\alpha$ 1AT deficiency. Twenty-one patients were given 60 mg of active plasma-derived  $\alpha$ 1AT per kilogram of body weight, once a week for up to six months. After a steady state had been reached, the group had trough serum levels of  $\alpha$ 1AT of  $126 \pm 1$  mg per deciliter as compared with  $30 \pm 1$  mg per deciliter before treatment, and serum anti-neutrophil elastase capacities of  $13.3 \pm 0.1$   $\mu$ M as compared with  $5.4 \pm 0.1$   $\mu$ M. The  $\alpha$ 1AT level in the epithelial lining fluid of the lungs was  $0.46 \pm 0.16$   $\mu$ M before treatment, and the anti-neutrophil elastase capacity was  $0.81 \pm 0.13$   $\mu$ M. Six days after infusion,  $\alpha$ 1AT levels ( $1.89 \pm 0.17$   $\mu$ M) and anti-neutrophil elastase capacities ( $1.65 \pm 0.13$   $\mu$ M) in the lining fluid were significantly increased ( $P < 0.0001$ ). Because of the chronicity of the disorder and the lack of sensitive measures of lung destruction, the clinical efficacy of this therapy could not be studied rigorously. No changes in lung function were observed in our patients over six months of treatment. The only important adverse reactions to the 507 infusions were four episodes of self-limited fever. This study demonstrates that infusions of  $\alpha$ 1AT derived from plasma are safe and can reverse the biochemical abnormalities in serum and lung fluid that characterize this disorder. Together with lifetime avoidance of cigarette smoking, replacement therapy with  $\alpha$ 1AT may be a logical approach to long-term medical treatment.

The null-null phenotype of  $\alpha$ 1AT, a phenotype characterized by no detectable  $\alpha$ 1AT in serum, presents a rare opportunity to examine the contribution of  $\alpha$ 1AT to the anti-neutrophil elastase protection of the lower respiratory tract. The subject, a 35-yr-old lifetime nonsmoker with moderate emphysema, has been characterized as having  $\alpha$ 1AT serum levels of zero resulting from the homozygous inheritance of  $\alpha$ 1AT genes that do not express detectable  $\alpha$ 1AT mRNA transcripts. Evaluation of the anti-neutrophil elastase capacity of the null-null serum showed it was  $< 5\%$  of normal, whereas that of the epithelial lining fluid (ELF) of the lower respiratory tract was  $13\%$  of normal. However, after 60 mg/kg of intravenously administered  $\alpha$ 1AT augmentation therapy once weekly for 4 wks, the serum  $\alpha$ 1AT levels peaked at  $> 300$  mg/dl, trough levels just prior to the next infusion were  $81 \pm 2$  mg/dl, and the average serum level integrated for the month of infusions was 138 mg/dl. Consistent with this serum rise in  $\alpha$ 1AT, the serum anti-neutrophil elastase capacity increased in parallel ( $r = 0.98$ ). Importantly, evaluation of the ELF 2 and 6 days after infusion demonstrated increases of  $\alpha$ 1AT levels (range 1.4 to 2.1  $\mu$ M) and anti-neutrophil elastase capacity (range 1.6 to 2.5  $\mu$ M), values within the lower range of normal. Furthermore, the lung ELF  $\alpha$ 1AT levels rose in direct proportion to the serum  $\alpha$ 1AT levels, and the ELF anti-neutrophil elastase capacity rose in direct proportion to the ELF  $\alpha$ 1AT levels. Taken together, these findings demonstrate that  $\alpha$ 1AT provides  $> 85\%$  of the anti-neutrophil elastase protection for the lower respiratory tract and that this protection can be modulated by the blood levels of  $\alpha$ 1AT.

Typically, inactivation of serine proteases by protein inhibitors involves the formation of a very stable bimolecular complex between the active sites of the inhibitor and the enzyme. The reaction mechanism and the structure



of the inhibition complex are well understood for several inhibitor classes, the most detailed analysis coming from X-ray crystallographic studies of the complexes. For the plasma protease inhibitor family, including  $\alpha$ 1AT, much less information is available. Attempts have been made to determine the 3-D structure of native  $\alpha$ 1AT but successful crystallography was achieved only with a proteolytically cleaved form which, because of a large conformational change, gave no information concerning the geometry of the active site. Nevertheless it is clear that the  $\alpha$ 1AT active site acts as a pseudosubstrate for the cognate protease and that the central P1 Met residue (position 358) plays a crucial role in the inhibition process. The importance of the P1 Met has been demonstrated by site-specific mutagenesis studies in which this residue was replaced by val recombinant  $\alpha$ 1AT (val<sup>358</sup>) produced in yeast or *Escherichia coli* retained elastase inhibitory capacity but, unlike the native form, was resistant to inactivation by chemical or biological oxidants. Thus the presence of Met sulfoxide at position 358 is sufficient to inactivate the molecule. Several studies have indicated that such oxidative inactivation occurs in the lungs of heavy cigarette smokers and therefore may be involved in the pathogenesis of acquired emphysema. Comparison of the active site sequences of the plasma protease inhibitor family, for example antithrombin III (ATIII),  $\alpha$ 1 antichymotrypsin and  $\alpha$ 1AT, shows that the residue of P1 corresponds to the specificity of the cognate protease. Moreover, direct evidence that the  $\alpha$ 1AT P1 residue is crucial in determining the specificity of inhibition was provided by the identification of a natural variant ( $\alpha$ 1AT Pittsburgh) which contains a P1 Met<sup>358</sup>  $\rightarrow$  Arg substitution. The single case described led to a fatal hemorrhagic disorder resulting from the potent thrombin inhibition capacity of the mutant inhibitor. Characterization of *E. coli*-produced recombinant  $\alpha$ 1AT (Arg<sup>358</sup>) showed that, in addition to thrombin, the P1 Met  $\rightarrow$  Arg substitution results in strong inhibition of other Arg-specific serine proteases, such as kallikrein and factor XII. We have investigated in more depth the effect of amino acid replacements at the P1 and subsite positions of the  $\alpha$ 1AT active site. Seven active site variants of human  $\alpha$ 1AT were produced in *Escherichia coli* following site-specific mutagenesis of the  $\alpha$ 1AT complementary DNA.  $\alpha$ 1AT (ala<sup>358</sup>),  $\alpha$ 1AT (ile<sup>358</sup>) and  $\alpha$ 1AT (val<sup>358</sup>) were efficient inhibitors of both neutrophil and pancreatic elastases, but not of cathepsin G.  $\alpha$ 1AT (ala<sup>358</sup>, val<sup>358</sup>) and  $\alpha$ 1AT (phe<sup>358</sup>) specifically inhibited pancreatic elastase and cathepsin G respectively. The most potent inhibitor of neutrophil elastase was  $\alpha$ 1AT (leu<sup>358</sup>), which also proved to be effective against cathepsin G. The  $\alpha$ 1AT(arg<sup>358</sup>) variant inactivated thrombin with kinetics similar to antithrombin III in the presence of heparin. Electrophoretic analysis showed that SDS-stable high molecular weight complexes were formed between the mutant inhibitors and the cognate proteases in each case. These data indicate that effective inhibition occurs when the  $\alpha$ 1AT P1 residue (position 358) corresponds to the primary specificity of the target protease. Moreover, alteration of the P3 residue (position 356) can further modify the reactivity of the inhibitor. Two of the variants have therapeutic potential:  $\alpha$ 1AT (leu<sup>358</sup>) may be more useful than plasma  $\alpha$ 1AT in the treatment of destructive lung disorders and  $\alpha$ 1AT (arg<sup>358</sup>) could be effective in the control of thrombosis.

The current concept relating to the pathogenesis of emphysema associated with cigarette smoking is that an imbalance exists within the lower respiratory tract between neutrophil elastase and the local anti-neutrophil



elastase screen, enabling uninhibited neutrophil elastase to destroy the alveolar structures over time. The possible role of the alveolar macrophage in contributing to this imbalance was investigated by evaluating the ability of cigarette smokers' alveolar macrophages to inactivate  $\alpha$ 1AT, the major anti-neutrophil elastase of the human lower respiratory tract. In vitro, alveolar macrophages of smokers spontaneously released 2.5-fold more superoxide anion and 8-fold more  $H_2O_2$  than macrophages of nonsmokers ( $p < 0.01$ , both comparisons). Using a model system which reproduced the relative amounts of alveolar macrophages and  $\alpha$ 1AT found in the epithelial lining fluid of the lower respiratory tract, we observed that smokers' macrophages caused a  $60 \pm 5\%$  reduction in the ability of  $\alpha$ 1AT to inhibit neutrophil elastase. In marked contrast, under the same conditions, non-smokers' macrophages had no effect upon the anti-neutrophil elastase function of  $\alpha$ 1AT. Addition of superoxide dismutase and catalase prevented inactivation of  $\alpha$ 1AT by smokers' macrophages, implying that the release of oxidants mediated the inactivation of  $\alpha$ 1AT. In addition, by utilizing a recombinant DNA produced modified form of  $\alpha$ 1AT containing an active site substitution (met<sup>358</sup>  $\rightarrow$  val), the inactivation of  $\alpha$ 1AT by smokers' alveolar macrophages was prevented, suggesting that the smokers' macrophages inactivate the  $\alpha$ 1AT by oxidizing the active site of the  $\alpha$ 1AT molecule. These results suggest that in cigarette smokers, the alveolar macrophage can modulate the activity of  $\alpha$ 1AT as an inhibitor of neutrophil elastase and thus play a role in the pathogenesis of emphysema associated with cigarette smoking.

To evaluate the potential use of recombinant DNA produced  $\alpha$ 1AT to augment the lung anti-neutrophil elastase defenses in  $\alpha$ 1AT deficiency, we compared the kinetics of intravenously administered recombinant produced  $\alpha$ 1-antitrypsin (r $\alpha$ 1AT) and purified normal human plasma  $\alpha$ 1-antitrypsin (p $\alpha$ 1AT) in the blood and lung of rhesus monkeys. The r $\alpha$ 1AT was produced in yeast transformed with an expressing plasmid containing a full length human  $\alpha$ 1-antitrypsin cDNA and purified to  $>99\%$  homogeneity. The r $\alpha$ 1AT has a MW of 45,000 daltons, no carbohydrates and is identical in sequence to normal plasma  $\alpha$ 1AT except for an additional N-terminal acetyl-methionine. Despite its lack of carbohydrates, the r $\alpha$ 1AT inhibited human neutrophil elastase with an association rate constant similar to that of p $\alpha$ 1AT. Rhesus monkeys were infused intravenously with 120 mg/kg of r $\alpha$ 1AT (n=13) or p $\alpha$ 1AT (n=12) and the serum, urine, and lung epithelial lining fluid (ELF) concentrations of these molecules quantified at various intervals. Although the initial serum levels of the r $\alpha$ 1AT and p $\alpha$ 1AT were both dose dependent, the p $\alpha$ 1AT remained in the blood for at least 4 days while the r $\alpha$ 1AT disappeared rapidly, such that it was barely detectable at 24 hr and undetectable thereafter. Strikingly, while no p $\alpha$ 1AT was detectable in the urine at any time, 38% of the intravenously administered r $\alpha$ 1AT was excreted within 3 hr. Similar to its behavior in man, the p $\alpha$ 1AT diffused into the lung such that its concentration in the ELF of the lower respiratory tract 1 to 4 days after infusion was approximately 10% that in serum. Interestingly, the r $\alpha$ 1AT molecule also diffused into the lung, with ELF levels at 24 hr similar to that of the p $\alpha$ 1AT. Furthermore, while the r $\alpha$ 1AT ELF levels declined by 48 hr and 96 hr to below those of the p $\alpha$ 1AT ELF levels, the r $\alpha$ 1AT ELF levels exceeded those in blood at the same time points. Importantly, like the p $\alpha$ 1AT, intravenous administration of r $\alpha$ 1AT resulted in a significant augmentation of the anti-neutrophil elastase capacity of





the ELF. Thus, in primates, human based r $\alpha$ 1AT has very different pharmacokinetics than does human p $\alpha$ 1AT, likely because of its modified charge and/or conformation. Despite this, however, the r $\alpha$ 1AT does diffuse into the lung and augments the anti-neutrophil elastase capacity of the epithelial lining fluid of the lower respiratory tract, suggesting it has potential as a therapeutic agent in the treatment of disorders such as  $\alpha$ 1AT deficiency.

Recombinant DNA directed human  $\alpha$ 1-antitrypsin (r $\alpha$ 1AT) functions identically to normal human  $\alpha$ 1AT as an elastase inhibitor but its lack of carbohydrate side chains and consequent short half-life following intravenous administration precludes its use in treating  $\alpha$ 1AT deficiency by intermittent intravenous infusion. Aerosol administration would target the r $\alpha$ 1AT appropriately and thus circumvents this problem, but it would be efficacious only if the r $\alpha$ 1AT reaches the alveoli and diffuses across the alveolar walls. To evaluate this approach, human  $\alpha$ 1AT cDNA-directed yeast-produced r $\alpha$ 1AT was administered intravenously or by aerosol to sheep (n=11) and the concentrations of r $\alpha$ 1AT over time measured in plasma, lung epithelial lining fluid (ELF), and lung lymph. Using a dose of 60 mg/kg, intravenous infusion resulted in lung ELF levels of  $400 \pm 100$  nM after 2 hrs. In contrast, using a nebulization system that generated >95% of particles  $\leq 5 \mu\text{m}$  and  $34 \pm 2\% \leq 2 \mu\text{m}$ , a dose of only 2.5 mg/kg of aerosolized r $\alpha$ 1AT resulted in the same ELF levels at 2 hrs ( $p > 0.1$ ). Importantly, the aerosolized r $\alpha$ 1AT appeared in lung lymph in a time dependent manner (1 hr  $2 \pm 1$  nM, 2 hr  $13 \pm 6$  nM, 3 hr  $27 \pm 17$  nM, 4 hr  $117 \pm 30$  nM), driven by a concentration gradient in ELF (2 hr  $400 \pm 50$  nM). Thus, r $\alpha$ 1AT can be aerosolized into sufficient size to access the alveolar spaces and in vivo reaches the epithelial surface and interstitium of the lower respiratory tract where it could augment the anti-elastase defenses, thus potentially useful for therapy of  $\alpha$ 1AT deficiency.

The concept of treatment is similar for all forms of  $\alpha$ 1AT deficiency: enhance the anti-neutrophil elastase protection in the lower respiratory tract. The current approach has been to augment levels by the infusion of  $\alpha$ 1AT purified from pooled human plasma. Alternatively, it should be possible to utilize a human  $\alpha$ 1AT cDNA to direct the production of  $\alpha$ 1AT. The normal human  $\alpha$ 1AT cDNA has been used to transform *Escherichia coli* and yeast, but the recombinant  $\alpha$ 1AT molecules produced lack carbohydrates; consequently, their plasma half-life is markedly reduced, obviating the intravenous route for their administration. As an approach to augment  $\alpha$ 1AT levels in this disorder with physiologically normal human  $\alpha$ 1AT, we have integrated a full-length normal human  $\alpha$ 1AT cDNA into the genome of mouse fibroblasts. To accomplish this, the retroviral vector N2 was modified by inserting the simian virus 40 early promoter followed by the  $\alpha$ 1AT cDNA. Southern analysis demonstrated that the intact cDNA was present in the genome of selected clones of the transfected murine fibroblasts  $\psi$ 2 and infected NIH/3T3. The clones produced three mRNA transcripts (5.8, 4.8, and 2.4 kilobases) containing human  $\alpha$ 1AT sequences, secreted on  $\alpha$ 1AT molecule recognized by an anti-human  $\alpha$ 1AT antibody, with the same molecular mass (52 kDa) as normal human  $\alpha$ 1AT and that complexed with and inhibited human neutrophil elastase. The  $\psi$ 2 produced  $\alpha$ 1AT was glycosylated, and when infused intravenously into mice, it had a serum half-life similar to normal  $\alpha$ 1AT purified from human plasma and markedly longer than that of



nonglycosylated human  $\alpha$ 1AT cDNA-directed yeast-produced  $\alpha$ 1AT. These studies demonstrate the feasibility of using a retroviral vector to insert the normal human  $\alpha$ 1AT cDNA into non- $\alpha$ 1AT-producing cells, resulting in the synthesis and secretion of physiologically "normal" human  $\alpha$ 1AT.

As a model approach to "gene therapy" of  $\alpha$ 1AT deficiency, a retroviral vector was used to insert the human  $\alpha$ 1AT cDNA into the genome of mouse fibroblasts to create a clonal population of mouse fibroblasts secreting human  $\alpha$ 1AT. After demonstrating that this clone of fibroblasts produced  $\alpha$ 1AT after more than 100 population doublings in the absence of selection pressure, the clone was transplanted into the peritoneal cavities of nude mice. When the animals were evaluated 4 weeks later, human  $\alpha$ 1AT was detected in both sera and the epithelial surface of the lungs. The transplanted clone of fibroblasts could be recovered from the peritoneal cavities of those mice and demonstrated to still be producing human  $\alpha$ 1AT. Thus, even after removal of selective pressure, a single clone of retroviral vector-infected cells that expressed an exogenous gene in vitro, continued to do so in vivo, and when recovered, continued to produce the product of the exogenous gene.

$\alpha$ 1AT, the major inhibitor of neutrophil elastase (NE) in the human lower respiratory tract (LRT), interacts with NE through the  $\alpha$ 1AT met<sup>358</sup>-ser<sup>359</sup> active site with an association rate constant (Kassoc) of  $10^7 \text{ M}^{-1}\text{sec}^{-1}$ . To evaluate the hypothesis that  $\alpha$ 1AT in the LRT of cigarette smokers is less capable of inhibiting NE in a time-dependent fashion than LRT  $\alpha$ 1AT of nonsmokers, the Kassoc of LRT  $\alpha$ 1AT for NE was compared in current smokers (n=7, 15 $\pm$ 4 pack-yr, normal lung function) and nonsmokers (n=12), normal lung function). To accomplish this,  $\alpha$ 1AT was purified (>95%) from lavage fluid using affinity and molecular sieve chromatography and the Kassoc for NE quantified using a time-dependent (0-7) min assay with equal amounts of  $\alpha$ 1AT and NE. The Kassoc of smoker plasma  $\alpha$ 1AT ( $9.5 \pm 0.5 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ ) was similar to that of nonsmoker plasma  $\alpha$ 1AT ( $9.3 \pm 0.7 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ , p>0.5). In marked contrast, the Kassoc of smoker LRT  $\alpha$ 1AT ( $6.5 \pm 0.4 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ ) was significantly lower (p<0.01) than that of nonsmoker LRT  $\alpha$ 1AT ( $8.1 \pm 0.5 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ ). Furthermore, the smoker LRT  $\alpha$ 1AT Kassoc was significantly less (p<0.01) than that of autologous plasma. But in the context of the concentration of  $\alpha$ 1AT in the LRT epithelial lining fluid, the inhibition time for NE of smoker LRT  $\alpha$ 1AT was 2-fold less than that of nonsmoker LRT  $\alpha$ 1AT (smoker:  $0.35 \pm 0.05 \text{ sec}$  vs nonsmoker:  $0.17 \pm 0.05 \text{ sec}$  p<0.01), i.e., for the concentration of  $\alpha$ 1AT in the LRT, it takes twice as long for an equivalent burden of NE to be inhibited in the smoker's lung compared to the non-smoker's lung. Thus, despite its presence in similar amounts, when evaluated in a kinetic fashion against its true target protease,  $\alpha$ 1AT in the lung of smokers was less able to provide a competent anti-neutrophil elastase screen than the  $\alpha$ 1AT of nonsmokers. These observations support the concept that cigarette smoking is associated with a shift in the balance of anti-neutrophil elastase-neutrophil elastase in the lung toward the elastase, making the lung more vulnerable to elastase and hence to destruction.

#### Significance to Biomedical Research

There are approximately 2 million individuals in the United States with



emphysema. Using  $\alpha$ 1AT deficiency as the "model" of emphysema, insights have been gained as to the pathogenesis of the destructive process in the lower respiratory tract. For the  $\alpha$ 1AT deficiency which represents approximately 2% of these individuals, replacement therapy with  $\alpha$ 1AT purified from pooled plasma is a reality. Within the next few years, it should be possible to evaluate the use of  $\alpha$ 1AT produced by recombinant DNA technology. With this background, it should be possible to develop an understanding of the pathogenesis and strategy for therapy to the acquired form of emphysema.

#### Proposed Course

Studies will be continuing to evaluate the pathogenesis of both hereditary and acquired forms of emphysema and to test therapies to suppress those processes that cause destruction of the lower respiratory tract.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

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## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanisms of Fibrosis

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The fibrotic lung disorders represent 15% of the non-infectious, non-malignant lung diseases; they are often progressive and can be fatal. The fibrosis results from damage caused by inflammatory cells and subsequent proliferation of mesenchymal cells, driven by mediators released by alveolar macrophages. The primary mediators are platelet-derived growth factor, fibronectin and alveolar macrophage derived growth factor. Other mediators include tumor necrosis factor. With knowledge of the specific processes involved, strategies can be developed to modulate these mediators as therapy for these disorders.

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Other Investigators: (continued)

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## Objectives

The current concepts of the mechanisms of pulmonary fibrosis hold that the accumulation of fibroblasts and fibroblast products results from two general processes. First, there must be damage to the alveolar wall; in most cases this is mediated by products of inflammatory cells, including macrophages, neutrophils and eosinophils. Second, there is enhanced proliferation of mesenchymal cells in the alveolar walls driven by growth signals released in the local milieu.

Although mononuclear phagocytes are known to be capable of releasing a variety of growth signals for fibroblasts, recent observations have demonstrated that one of these molecules is platelet-derived growth factor (PDGF) a 31 kDa dimeric glycoprotein that is potent both as a chemoattractant for mesenchymal cells as well as a stimulus for mesenchymal cells to enter the G1 phase of the cell cycle. Blood monocytes do not normally release PDGF, but do so when stimulated. When matured in culture, monocytes spontaneously release PDGF and tissue macrophages recovered from internal organs spontaneously release low levels of this potent mediator. The interest in PDGF as an important mediator of normal and pathologic scar formation has been heightened by the observation that the B-chain of PDGF is coded for by the *c-sis* proto-oncogene, a 30 kb gene localized to region q11>qtr of chromosome 22. Interestingly, while the *v-sis* oncogene (the retroviral analog of the human *c-sis* gene) was originally recovered from a naturally occurring sarcoma of a Woolly monkey and is capable of transforming normal fibroblasts into continuously proliferating cells *in vitro*, recent studies have shown that activated blood monocytes, *in vitro* matured monocytes, and alveolar macrophages all express the *c-sis* proto-oncogene as well as produce and secrete PDGF. Together, these observations support the concept that, in addition to a possible role in malignancy, activation of the *c-sis* proto-oncogene could play a key role in the pathogenesis of tissue fibrosis. To evaluate this concept, alveolar macrophages were recovered from patients with idiopathic pulmonary fibrosis (IPF), a fibrotic disorder characterized by replacement of the normal alveolar walls by scar tissue dominated by the accumulation of mesenchymal cells. Since normal alveolar macrophages express *c-sis* proto-oncogene and release low levels of PDGF, and since IPF is likely initiated by immune complexes activating alveolar macrophages in the lower respiratory tract, we hypothesized that normal alveolar macrophages stimulated with immune complexes would be stimulated to release increased amounts of PDGF and alveolar macrophages recovered from individuals with IPF would spontaneously release exaggerated amounts of active PDGF. Evaluation of normal alveolar macrophages stimulated with immune complexes demonstrated several-fold increase in PDGF release. Importantly, evaluation of alveolar macrophages recovered from the lungs of IPF patients demonstrated spontaneous release of 4-fold more PDGF than alveolar macrophages recovered from normal individuals. Furthermore, these PDGF molecules were active, as evidenced by their chemotactic activity for smooth muscle cells and ability to act as a "competence" factor for fibroblast growth. Together, these observations suggest the possibility that mesenchymal cell accumulation within the alveolar walls in this fibrotic disorder may result, in part, from the exaggerated release of PDGF by mononuclear phagocytes in the lower respiratory tract.



Since normal human platelet PDGF molecules are dimers of A-and B-chains and platelets and monocytes are derived from the same marrow precursors, it is possible that in addition to the PDGF B-chain (the c-sis gene product), mononuclear are also capable of expressing the PDGF A-chain. To evaluate this concept, activated blood monocytes were simultaneously evaluated for the expression of PDGF A-and B-chain genes. Human blood monocytes were purified and cultured with and without activation by lipopolysaccharide and poly(A)+ RNA evaluated using Northern analysis and <sup>32</sup>P-labeled A-chain and B-chain (human c-sis) probes. Unstimulated blood monocytes did not express either A-chain or B-chain genes. In contrast, activated monocytes expressed a 4.2 kb mRNA B-chain transcript at 4 hr, but the B-chain mRNA levels declined significantly over the next 18 hr. In comparison, activated monocytes expressed very little A-chain mRNA at 4 hr, but at 12 hr 1.9, 2.3, and 2.8 kb transcripts were observed and persisted through 24 hr. Thus, activation of blood monocytes is followed by PDGF B-chain gene expression preceding PDGF A-chain gene expression, suggesting a difference in the regulation of the expression of the genes for these two chains by these cells.

Fibronectin is a 440,000 dalton dimeric glycoprotein produced by a variety of cells and capable of functioning in a broad range of biologic processes including cell adhesion, spreading, cytoskeletal organization, migration, proliferation and differentiation. Fibronectin accomplishes these diverse processes by acting as a ligand between cells and macromolecules through the use of structural domains in the fibronectin molecule with affinity for collagen, heparin, fibrin, DNA and cell surfaces. Several studies have demonstrated that alveolar macrophages synthesize and secrete fibronectin and that alveolar macrophages associated with chronic inflammatory diseases of the lower respiratory tract secrete exaggerated amounts of this molecule. In the context of the known functions of fibronectin, it is likely that it represents one of the alveolar macrophage mediators that plays a role in both defending the lung and in modulating the damage and repair that accompanies inflammatory processes in the lower respiratory tract. To gain insight into the processes that modulate the level of fibronectin secretion by these cells, monocytes, in vitro matured monocytes and alveolar macrophages were used as models to compare fibronectin mRNA levels and fibronectin secretion in a variety of circumstances. Using Northern analysis and dot-blot analysis with a <sup>32</sup>P-labeled human fibronectin cDNA probe, steady state mRNA levels were evaluated. Human fibronectin specific enzyme-linked immunoassay was used to evaluate fibronectin secretion. In all cases the amounts of fibronectin secreted paralleled fibronectin mRNA levels. Specifically: (1) when fibronectin mRNA was undetectable, as in the case of normal blood monocytes, no fibronectin was secreted, but whenever fibronectin mRNA was present, as in normal alveolar macrophages, fibronectin was secreted by the cells; (2) as monocytes matured into macrophages in vitro, the cells began to express fibronectin mRNA and the cells secreted fibronectin; (3) when alveolar macrophages were activated with surface stimuli such as lipopolysaccharide (LPS) or immune complexes, fibronectin mRNA levels decreased and in parallel, the cells secreted less fibronectin; (4) in IPF, alveolar macrophages contained several fold more fibronectin mRNA transcripts than normal and the cells spontaneously secreted several fold more fibronectin than normal; and (5) when IPF alveolar macrophages were placed in culture, the fibronectin mRNA levels in





the cells decreased with time, and concurrently, the amounts of fibronectin produced per unit time continually decreased. The observation of a strict concordance of fibronectin mRNA levels and fibronectin release by mononuclear phagocytes, suggests that, at least in many circumstances, fibronectin secretion by mononuclear phagocytes is controlled by steady state levels of fibronectin mRNA.

In the context of the observations that the amount of fibronectin produced by mononuclear phagocytes parallels the average levels of fibronectin mRNA in these cells, a study was designed to evaluate whether the steady state fibronectin mRNA levels observed in a population of alveolar macrophages reflect the fibronectin mRNA levels for all macrophages in the population, or whether fibronectin mRNA levels vary from cell to cell. To approach this question, we utilized *in situ* hybridization techniques with fibronectin RNA probes to evaluate the proportion of alveolar macrophages expressing fibronectin mRNA transcripts to determine whether there are differences in the morphological characteristics of the macrophages expressing or not expressing the fibronectin gene, and, for those macrophages expressing the fibronectin gene, whether there are differences in the number of fibronectin mRNA copies per cell. As models for circumstances in which *in vivo* fibronectin expression may differ on a cell-to-cell basis, we utilized alveolar macrophages from normal individuals and from patients with IPF. Utilizing *in situ* hybridization of  $^{35}\text{S}$ -labeled anti-sense and sense RNA fibronectin probes, the study demonstrated that most, but not all, normal alveolar macrophages contain fibronectin mRNA transcripts, and that among those macrophages expressing this gene, the relative amount of fibronectin mRNA transcripts varies from cell to cell. Interestingly, while  $66\pm 3\%$  of normal alveolar macrophages contain fibronectin mRNA transcripts, this is increased to  $82\pm 2\%$  ( $p < 0.01$ ) of alveolar macrophages recovered from the lungs of individuals with IPF, a disorder associated with exaggerated amounts of fibronectin in the lower respiratory tract. Furthermore, of the macrophages expressing the fibronectin gene, those from IPF patients contain more fibronectin mRNA transcripts than those from normals. Consistent with this observation, evaluation of tissue samples from IPF patients demonstrated that of all cells present, alveolar macrophages showed the greatest numbers of fibronectin mRNA transcripts per cell. These observations demonstrate that there can be marked cell-to-cell variation in the expression of the gene for a macrophage product such as fibronectin, suggesting that there are processes that modulate similar cells in the same anatomic compartment to vary their expression of the same gene.

Although alveolar macrophages are thought to play an important role in fibrogenesis by releasing mediators that have the capacity to recruit mesenchymal cells and stimulate them to proliferate, under resting conditions, lung fibroblast renewal proceeds slowly, suggesting that suppressive signals may be operating in the milieu of the lower respiratory tract to prevent uncontrolled mesenchymal cell accumulation. In this regard, we hypothesized that alveolar macrophages may express the gene for transforming growth factor- $\beta$  (TGF- $\beta$ ), a 25 kd dimeric protein that suppresses the growth response of many normal cells. To evaluate this hypothesis, RNA was recovered from alveolar macrophages of normal individuals, and evaluated for the presence of TGF- $\beta$  mRNA using Northern-analysis and a  $^{32}\text{P}$ -labeled TGF- $\beta$  probe. Autoradiograms revealed that resting alveolar macrophages



contained 2.5 kb TGF- $\beta$  specific mRNA transcripts, i.e., resting alveolar macrophages constitutively express the TGF- $\beta$  gene. Interestingly, when the alveolar macrophages were stimulated with LPS, a classical "model" stimulant of inflammation, there was no upregulation of the TGF- $\beta$  gene. In contrast to the manner in which the TGF- $\beta$  was modulated, the LPS stimulated macrophages produced more PDGF, a potent mitogen for fibroblast proliferation. Thus, the resting and stimulated cells expressed similar amounts of the TGF- $\beta$  gene, but the stimulated cells produced far more of the positive growth signal PDGF. Consistent with the concept of a suppressive role for TGF- $\beta$ , over an 18-hr period in serum free conditions, purified recombinant TGF- $\beta$  was able to suppress PDGF + insulin driven proliferation of human lung fibroblasts. In this context, by releasing TGF- $\beta$  into the local milieu of the lower respiratory tract, resting alveolar macrophages may play a role in suppression of uncontrolled mesenchymal cell accumulation. However, when activated, the alveolar macrophages armamentarium of positive growth signals is probably sufficient to override the suppressive effect of TGF- $\beta$ .

Among the mediators that mononuclear phagocytes are capable of producing is tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; also called "cachectin"), a multimeric protein consisting of 17 kd subunits that is cytotoxic for tumors, has growth activity for fibroblasts, and has systemic effects such as fever and cachexia. To evaluate the control of TNF- $\alpha$  expression by mononuclear phagocytes, we investigated the ability of normal human blood monocytes and alveolar macrophages to express the TNF- $\alpha$  gene and secrete TNF- $\alpha$ . Evaluation of mononuclear phagocyte mRNA by Northern analysis with a  $^{32}\text{P}$ -labeled TNF- $\alpha$  cDNA probe demonstrated that resting monocytes and alveolar macrophages expressed very little TNF- $\alpha$  mRNA. In contrast, when stimulated with LPS, both blood monocytes and alveolar macrophages demonstrated the 1.8 kb mRNA transcripts specific for TNF- $\alpha$ . In parallel, supernatants from monocytes and alveolar macrophages cultured for 24 hr with LPS had TNF- $\alpha$  activity as demonstrated by cytotoxicity of mouse L-929 fibroblasts that was abrogated by a specific anti-TNF- $\alpha$  antibody and was markedly suppressed when the LPS-stimulated mononuclear cells were incubated in the presence of cycloheximide. This mononuclear phagocyte cytotoxic activity co-eluted on a molecular sieve with recombinant human TNF- $\alpha$ , further identifying it as TNF- $\alpha$ . Interestingly, quantification of the TNF- $\alpha$  activity released by monocytes and alveolar macrophages demonstrated that, on a per cell basis using a standard LPS stimulus, alveolar macrophages released 7-fold more TNF- $\alpha$  than did blood monocytes. Thus, while both monocytes and alveolar macrophages are capable of expressing the TNF- $\alpha$  gene when stimulated, alveolar macrophages appear to be more capable as TNF- $\alpha$  producers. This capacity is in contrast to the relatively poor capacity of the alveolar macrophages to produce interleukin-1, a mediator with a very similar spectrum of activity.

One prominent feature of IPF is the marked changes to the alveolar epithelial cells, including destruction of type I alveolar epithelial cells and repopulation of the epithelial surface by type II alveolar epithelial cells and bronchiolar epithelial cells. Alveolar macrophages and neutrophils dominate the inflammatory cell population in the lower respiratory tract of patients with IPF. Because both cell types are capable of inducing oxidant-mediated lung parenchymal cell cytotoxicity, one mechanism to



explain the epithelial cell injury associated with IPF is the spontaneous release of toxic oxidants by alveolar macrophages and neutrophils. In addition, when activated, neutrophils can also release myeloperoxidase, a 115 kDa protein which can interact with  $H_2O_2$ , a product of both mononuclear phagocytes and neutrophils, to form the highly toxic hypohalide anion. In this regard, it is reasonable to hypothesize that the alveolar epithelial cell injury that characterizes IPF may result, at least in part, from an enhanced oxidant burden that may exist in the lower respiratory tract of these patients. To evaluate this concept, inflammatory cells recovered by bronchoalveolar lavage from the lower respiratory tract of patients with IPF were evaluated for their ability to spontaneously cause oxidant-mediated alveolar epithelial cell cytotoxicity in the presence and absence of epithelial lining fluid (ELF) from the same individuals. The IPF cells were spontaneously releasing exaggerated amounts of superoxide ( $O_2^-$ ) and  $H_2O_2$  compared with normal ( $p < 0.02$ ). Cytotoxicity to the alveolar epithelial (AKD) cells was markedly increased when IPF inflammatory cells were incubated with autologous ELF ( $p < 0.02$ ). The majority of IPF patients had ELF myeloperoxidase levels above normal ( $p < 0.002$ ). Incubation of IPF ELF with AKD cells in the presence of  $H_2O_2$  caused increased cellular injury ( $p < 0.01$  compared with control), which was suppressed by methionine, a myeloperoxidase system scavenger. IPF patients with high concentrations of ELF myeloperoxidase deteriorated more rapidly than those with low ELF myeloperoxidase ( $p < 0.05$ ). Thus, IPF is characterized by an increased spontaneous production of oxidants by lung inflammatory cells, the presence of high concentrations of myeloperoxidase in the ELF of the lower respiratory tract, and a synergistic cytotoxic effect of alveolar inflammatory cells and ELF on lung epithelial cells, suggesting oxidants may play a role in causing the epithelial cell injury of this disorder.

Although lung parenchymal cells have an array of intracellular anti-oxidants to protect themselves from toxic oxidants, much of the oxidant burden imposed on the lower respiratory tract is brought to the extracellular milieu of the parenchymal cells by activated inflammatory cells, inhaled gases, and xenobiotics. In this context, we hypothesized that the extracellular milieu of the alveolar structures may contain macromolecules with antioxidant properties which serve as a first line of defense against oxidants generated outside of the parenchymal cells. To evaluate this concept, a model of extracellular oxidant mediated lung parenchymal cell injury was developed using  $^{51}Cr$ -labeled human lung fibroblasts (HFL-1) exposed to a  $H_2O_2$  generating system (glucose, glucose oxidase) for 8 hr,  $37^\circ$ . Using this model, alveolar epithelial lining fluid (ELF) obtained from healthy nonsmokers by bronchoalveolar lavage was tested for its ability to protect lung parenchymal cells against injury initiated by  $H_2O_2$ . The ELF provided marked antioxidant protection to the lung cells when incubated with the  $H_2O_2$  generating system. Characterization of the antioxidant properties of the macromolecules in ELF demonstrated its anti- $H_2O_2$  defenses were provided mostly by a  $H_2O$  soluble fraction with a molecular weight in the range of 100 to 300 kDa. Although the plasma proteins ceruloplasmin and  $\alpha_1$ -antitrypsin can provide anti- $H_2O_2$  protection and are present in ELF, these macromolecules are in insufficient concentrations to provide the antioxidant protection observed in normal ELF. However, catalase, a normal intracellular antioxidant, was present in normal ELF in sufficient concentration to account for most of the observed anti- $H_2O_2$  properties of ELF.



Furthermore, when ELF was depleted of catalase with an anti-catalase antibody, the anti-H<sub>2</sub>O<sub>2</sub> macromolecular defenses of ELF were abolished. Although the source of the catalase is unknown, it was not derived from red blood cells and evaluation of its concentration relative to other intracellular antioxidants and its isoenzyme pattern suggested that its presence was not due to an artifact of the lavage procedure and at least in part, cells other than inflammatory cells contribute to its presence. Since catalase is not normally released by cells, a likely explanation for its presence in high concentrations in normal ELF is that it is released by lung inflammatory and parenchymal cells onto the epithelial surface of the lower respiratory tract during their normal turnover and collects there due to the slow turnover of ELF. However, independent of its source or mechanisms of accumulation, it is likely that catalase in the ELF of the lower respiratory tract of normal individuals plays a role in protecting lung parenchymal cells against oxidants present in the extracellular milieu.

We have also evaluated ELF for the presence of glutathione (L-γ-glutamyl-L-cysteinyl-glycine), another antioxidant that might contribute to the antioxidant protection of the epithelial surface of the lung. The theoretical basis for this hypothesis rests in the knowledge that, although glutathione is a major component of intracellular antioxidant defenses, it is exported extracellularly. Specifically, (1) among the cell types known to export glutathione are mononuclear phagocytes, lymphocytes and fibroblasts, cells present in the lower respiratory tract; (2) although peripheral blood plasma levels of glutathione are very low, the glutathione concentration in hepatic vein plasma is much higher, consistent with the concept that the concentrations of glutathione may vary at different sites in the body; (3) γ-glutamyl-transpeptidase, a cell surface enzyme that functions to remove glutathione from the extracellular space, is present in the lung, but in far lower concentrations in the lung parenchyma than in the kidney, the major site of glutathione removal from plasma; and (4) although the epithelial lining fluid of the lower respiratory tract is replenished constantly, this process is relatively sluggish so that materials released into this compartment likely remain in the local milieu for some time. Direct evaluation by lavage demonstrated the total glutathione (the reduced form GSH and the disulfide GSSG) concentration of normal ELF was 140-fold higher than that in plasma of the same individuals, and 96% of the glutathione in ELF was in the reduced form. Compared with nonsmokers, cigarette smokers had 80% higher levels of ELF total glutathione, 98% of which was in the reduced form. Studies of cultured lung epithelial cells and fibroblasts demonstrated that these concentrations of reduced glutathione were sufficient to protect these cells against the burden of H<sub>2</sub>O<sub>2</sub> in the range released by alveolar macrophages removed from the lower respiratory tract of nonsmokers and smokers, respectively, suggesting that the glutathione present in the alveolar ELF of normal individuals likely contributes to the protective screen against oxidants in the extracellular milieu of the lower respiratory tract.

The chronic inhalation of inorganic dust in an occupational setting can result in the development of interstitial lung disease, a chronic disorder of the lower respiratory tract associated with dyspnea and a limitation in the transfer of oxygen from air to blood. Referred to as "pneumoconioses," the common inorganic dust diseases are those resulting from the chronic





exposure to high concentrations of airborne asbestos ("asbestosis"), coal ("coal workers' pneumoconiosis," CWP), and silica ("silicosis"). There has been extensive study of the clinical manifestation of these disorders, and they are each well defined in terms of their associated symptomatology, physical findings, roentgenographic appearance, and physiologic dysfunction. However, despite the available epidemiologic and clinical information regarding these disorders, little is known of the processes occurring within the lower respiratory tract that are responsible for the derangements to the lung parenchyma that characterize these diseases. In this context, we assessed differences in cell populations and macrophage-derived mediator release within the lower respiratory tract of individuals with asbestosis, CWP, and silicosis. Specifically, we focused on the mediators being released by the chronic inflammatory cells in the lower respiratory tract that may play a role in mediating injury and fibrosis of the lung parenchyma in these disorders. In all 3 disorders, the inflammation was dominated by alveolar macrophages. Since a common feature of these interstitial lung diseases is concurrent injury and fibrosis of alveolar walls, we assessed whether these alveolar macrophages were spontaneously releasing mediators capable of giving rise to these changes. Alveolar macrophages from the study population were spontaneously releasing increased amounts of superoxide anion and hydrogen peroxide (both  $p < 0.01$  compared to normals), oxidants capable of injuring lung parenchymal cells. The alveolar macrophages were also spontaneously releasing significantly increased amounts of fibronectin and alveolar macrophage-derived growth factor (both  $p < 0.01$  compared to normals), mediators that act synergistically to signal fibroblast replication. Taken together, these findings define a major role for the alveolar macrophage in mediating the alveolar wall injury and fibrosis that characterize the common pneumoconioses, and suggest the alveolar macrophage is an important "target" for developing strategies designed to prevent loss of lung function in these individuals.

Chronic asbestos exposure is associated with the accumulation of mononuclear phagocytes in the lower respiratory tract. This process can be both protective and injurious, since macrophages can aid in asbestos clearance yet also modulate structural derangements of the alveolar walls. To understand why macrophages accumulate in the lungs of asbestos-exposed persons, two possible mechanisms were evaluated using alveolar macrophages from subjects with histories of chronic high exposure to airborne asbestos: (1) enhanced recruitment of blood monocytes to the lung; and (2) an increased rate of replication of macrophages in situ. Monoclonal antibody analysis with antibodies that detect surface antigens on the majority of circulating blood monocytes but only on a minority of mature alveolar macrophages demonstrated that an increased proportion of alveolar macrophages of asbestos workers expressed monocyte lineage antigens, suggesting the presence of "young" newly recruited macrophages and thus enhanced recruitment. Culture of the alveolar macrophages from these subjects with [ $^3$ H]thymidine followed by autoradiography demonstrated an increased proportion of alveolar macrophages synthesizing DNA, suggesting the macrophages are replicating at an increased rate in situ. These observations are consistent with the concept that both enhanced recruitment of blood monocytes and increased local proliferation of alveolar macrophages contribute to the accumulation of mononuclear phagocytes to the lung of persons with chronic asbestos exposure.



Langerhans' cells represent a distinct population of bone marrow derived cells associated with the mononuclear phagocyte system. Typically, these cells are 15 to 25  $\mu\text{m}$  in diameter, have a lobulated nucleus and, by ultrastructural analysis, demonstrate characteristic intracytoplasmic structures called Birbeck granules. Normally found in squamous epithelia such as the skin and in lymphoid organs such as the spleen, lymph nodes and thymus, Langerhans' cells are thought to function as antigen presenting cells. As such, Langerhans' cells are capable of phagocytosis, express HLA class II antigens and are capable of releasing interleukin-1. Langerhans' cells are relatively rare in the normal human lung, occasionally being found in the alveolar interstitium and bronchial walls; Langerhans' cells are not normally present on the pulmonary epithelial surface and are not normally found among the mononuclear phagocyte populations recovered by bronchoalveolar lavage. In marked contrast, Langerhans' cells occupy a prominent place among the cells accumulating in the lower respiratory tract in histiocytosis X, a disorder characterized by focal granulomatous lesions centered about the terminal bronchioles. Consistent with this knowledge, Langerhans' cells are commonly observed among the cells recovered by lavage of these patients. Although the etiology of pulmonary histiocytosis X is unknown, it is a striking epidemiologic observation that greater than 95% of affected individuals have a history of cigarette smoking. In this context, and with the knowledge that Langerhans' cells dominate the cellular lesions of histiocytosis X yet are very rare in the normal lung, we hypothesized that cigarette smoking may, in some manner, be associated with an expansion in the population of Langerhans' cells in the lung. To evaluate this hypothesis, we have examined cells recovered by bronchoalveolar lavage of normal nonsmokers and normal cigarette smokers for the presence of Langerhans' cells using both the monoclonal antibody OKT6, an antibody that recognizes Langerhans' cells, and transmission electron microscopy. While the OKT6 antibody identified  $0.1 \pm 0.1\%$  of the recovered cells in the nonsmokers, it labeled  $1.1 \pm 0.3\%$  recovered cells of the smokers ( $p < 0.01$ ). Furthermore, while electron microscopy demonstrated no Langerhans' cells among the lavage cells from nonsmokers,  $0.4 \pm 0.1\%$  of the cells recovered from normal smokers contained characteristic intracytoplasmic Birbeck granules, positively identifying them as Langerhans' cells. We conclude that cigarette smoking is associated with an expansion in the population of Langerhans' cells on the epithelial surface of the lower respiratory tract. While the mechanisms underlying this accumulation are unknown, it is possible that the properties of these cells contribute to the derangements of the pulmonary parenchyma found in cigarette smoking and establish a biological link to the already observed epidemiological association between histiocytosis X and cigarette smoking.

#### Significance to Biomedical Research and the Program of the Institute

The fibrotic lung disorders are often progressive and can be fatal. Over all, they represent approximately 15% of the non-infectious, non-malignant lung disorders in the United States. By understanding the processes by which the fibrosis occurs, it should be possible to design rational strategies to stage and treat these disorders.



Proposed Course

Work over the next several years will involve isolation of the specific genes and their products involved in driving the accumulation of fibroblasts in the lung parenchyma. As each of these mechanisms become apparent, strategies will be developed to attempt to modulate these genes in clinical studies.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

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## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

T-Lymphocyte Disorders

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The T-lymphocyte lung disorders occur in 20 to 50 per 100,000 of the U.S.A. population. The "model" disorder of this group is sarcoidosis, a disease characterized by the accumulation of activated helper T-lymphocytes at sites of disease. These T-cells spontaneously express the interleukin-2 gene, thus driving T-cells in the local milieu to proliferate. Treatment of these individuals with corticosteroids results in suppression of interleukin-2 gene expression, cessation of lung T-cell proliferation, and improvement in lung function.

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Pulmonary sarcoidosis is a disorder of the lower respiratory tract characterized by chronic inflammation, granuloma formation and, in some individuals, parenchymal fibrosis. Together these processes derange the alveoli, airways and blood vessels, consequently impairing the ability of the lung to exchange gas in the normal fashion. As with the other interstitial lung disorders, it is recognized that the inflammation precedes the other abnormalities that characterize this disorder. The inflammation of active pulmonary sarcoid is dominated by an accumulation of T-helper lymphocytes in the lung parenchyma. These T-cells are thought to play a central role in the pathogenesis of sarcoidosis in two ways. First, the accumulated T-cells distort the architecture of the parenchyma, thus altering the intimate relationships between air and blood. Second, the T-cell populations are activated and spontaneously releasing monocyte chemotactic factor and interferon-gamma, mediators that recruit and activate mononuclear phagocytes, respectively, events that are early steps in the process of granuloma formation. In this context, an understanding of the pathogenesis of pulmonary sarcoidosis is intimately linked to the understanding of the process directing the accumulation of T-lymphocytes in the lower respiratory tract of individuals with active disease. Relevant to this question, prior work from this laboratory has demonstrated that the T-lymphocytes recovered from the lungs of these patients are spontaneously proliferating and spontaneously releasing interleukin-2 (IL-2), the T-cell growth factor. Further analysis demonstrated that it is the activated HLA-DR+ helper (CD4+) T-cells which release the majority of the IL-2. Thus, while the stimulus that initiates the process is unknown, the IL-2 releasing lung T-cells are thought to be responsible for maintaining the T-cell inflammation and thus maintaining the disease in an active state.

To determine whether the IL-2 gene is activated in sarcoid T-cells in a systemic manner or only at sites of disease, cells obtained by bronchoalveolar lavage of individuals with active sarcoidosis, inactive sarcoidosis, and normals were evaluated for the spontaneous presence of IL-2 transcripts by using a human IL-2 cDNA probe and Northern analysis of extracted RNA. Freshly recovered lung cells of individuals with active pulmonary sarcoidosis contained 0.85 kb IL-2 mRNA transcripts. However, IL-2 mRNA transcripts could not be detected in fresh autologous blood T-cells or in purified autologous blood CD4+ T-cells, although IL-2 mRNA transcripts were inducible in these cells by phytohemagglutinin/phorbol myristate acetate. The sarcoid lung T cells, however, did not express the IL-2 gene constitutively; when placed in culture with no stimulation and evaluated after 24 hr, they demonstrated down regulation of the amounts of IL-2 mRNA transcripts, despite the fact that they were capable of re-expressing the IL-2 gene and releasing more IL-2 in response to added activation signals. Thus the activation of the IL-2 gene in T-cells in active sarcoidosis occurs at the site of disease and is not a generalized property of T-cells throughout the body, and is not sustained if the T-cells are removed from the sites of disease. Although the cause of sarcoid is unknown, these observations are consistent with the concept that sarcoid is associated with local stimuli at the site of disease inducing the CD4+ T cell IL-2 gene activation that plays such a critical role in the pathogenesis of this disease.

Since active pulmonary sarcoidosis is characterized by the alveolar accumulation of activated helper T-lymphocytes which are spontaneously releasing



IL-2 and proliferating at an enhanced rate, sarcoidosis represents a "model" human disorder to test in vivo the known in vitro action of corticosteroids on suppressing the activated IL-2 gene. To evaluate this concept, comparable groups of patients with active sarcoidosis were prospectively evaluated with no therapy or treated with corticosteroids. Over  $3.2 \pm 0.4$  months, the untreated group had no significant change in spontaneous lung T-cell release of IL-2 or spontaneous proliferation. In contrast, over the same period, the treated group had marked reduction of spontaneous lung T-cell release of IL-2 and proliferation ( $p < 0.01$ , all comparisons prior to therapy). Furthermore, Northern analysis of lung T-cell RNA prior to therapy demonstrated IL-2 mRNA transcripts while no IL-2 transcripts were observed during therapy. These observations are consistent with the concept that directly, or indirectly, corticosteroids are capable of suppressing the IL-2 gene in activated T-lymphocytes in vivo.

The exaggerated T-helper/inducer processes observed in sarcoid are in striking contrast to normal cellular immune responses, in which the initial accumulation of T-helper/inducer cells is followed by accumulation of T-suppressor/cytotoxic cells, a process associated with the damping of the overall immune response. In this regard, the exuberant T-helper/inducer processes in sarcoidosis could be explained by hypothesizing the presence of stimuli specific for helper/inducer T-cells that do not stimulate suppressor/cytotoxic T-cells and/or processes that specifically suppress suppressor/cytotoxic T-cells. Relevant to these concepts, it is known that individuals with sarcoidosis have circulating anti-T-cell antibodies, primarily of the IgM class. In this context, and in the context that active pulmonary sarcoidosis is characterized by the activation of T-helper/inducer cells in the lung without a concomitant increase in T-suppressor/cytotoxic cells, we evaluated these antibodies for a possible functional role in the pathogenesis of sarcoidosis. The strategy to examine this question was to evaluate serum and lavage fluid of active sarcoid patients for the presence of anti-T-lymphocyte antibodies, identify the target T-cell subset for these antibodies and evaluate these antibodies for possible stimulatory effects on T-helper/inducer cells and/or inhibitory effects on T-suppressor/cytotoxic cells. Indirect immunofluorescence studies demonstrated that sarcoid patients had anti-T-cell antibodies of the IgM type reacting with autologous as well as non-autologous normal T-cells. IgM recovered in sarcoid lavage fluid also reacted with T-cells, thus demonstrating the autoantibodies at the site of disease. Two color immunofluorescence and flow cytometry showed that these sarcoid autoantibodies bound to mostly CD8+ T-suppressor/cytotoxic cells, but also to a small proportion of CD4+ T-helper/inducer cells. Incubating lymphocytes with sarcoid serum or IgM purified from sarcoid serum did not stimulate T-cell proliferation. Furthermore, when CD8+ T-cells were stimulated with irradiated allogenic B-cells, increasing concentrations of sarcoid serum had no inhibitory effects on the activation and proliferative response of the CD8+ T-cells. Likewise, the purified IgM anti-T-cell antibodies had no inhibitory effects on the mitogenic response of CD8+ T-cells to the anti-T-cell antigen receptor-associated T3 complex antibody OKT3. Thus, the IgM anti-T-cell autoantibodies of sarcoidosis are present at the site of disease and bind to potentially relevant T-cell subsets, but there is no evidence that they have an identifiable functional role in the development



of the excess T-helper cell activity of sarcoidosis.

One clue to the mechanisms responsible for the exaggerated CD4+ T-cell inflammatory process in the sarcoid lung relates to the observation that the activation of lung CD4+ T-cell population occurs in the absence of comparable activation of lung CD8+ T-cells. In this context, we hypothesized that CD8+ T-suppressor/cytotoxic cells in sarcoid may have an impaired ability to respond to activation stimuli and to generate effector cells capable of suppressing the activation and proliferation of the helper/inducer T-cell population at the site of disease. If this occurs, it would help to explain why the spontaneous down-regulation of activated T-helper/inducer processes that characterizes normal immune responses is not observed in active pulmonary sarcoidosis. To evaluate this concept, a study was designed to assess the response of sarcoid CD8+ T-cells for their ability to express IL-2 receptors and proliferate in response to an activation signal and the ability of antigen activated CD4+ T-cells to activate autologous CD8+ T-cells to function to suppress antigen-specific CD4+ T-cell proliferation. Stimulation of purified sarcoid blood CD8+ T cells with the anti-T3/Ti complex monoclonal antibody OKT3 was followed by the normal expression of IL-2 receptor ( $p > 0.1$ ) and proliferation ( $p > 0.1$ ). In addition, lung sarcoid CD8+ T-cells responded to OKT3 similarly to autologous lung CD4+ T-cells as regards expression of IL-2 receptor ( $p > 0.1$ ) and proliferation ( $p > 0.1$ ). Finally, using CD4+ cells activated with allogenic antigen to induce, in co-culture, fresh autologous CD8+ cells to suppress proliferation of fresh autologous CD4+ cells to the same antigens, sarcoid CD8+ T-cells suppressed CD4+ cell proliferation in a normal fashion ( $p > 0.1$ ). These results demonstrate that sarcoid CD8+ (suppressor/cytotoxic) T-cells are competent to respond to a proliferation signal normally and can be induced to normally suppress CD4+ T-cell proliferation to antigens, suggesting that the expansion of activated CD4+ T-cells in pulmonary sarcoidosis is neither due to a generalized abnormality of CD8+ T-cells nor of their suppressor T-cell function.

One mechanism to explain the activated T-cell processes in sarcoid is to hypothesize that the T-cells in affected organs are "driven" to be activated in an exaggerated fashion by mononuclear phagocytes in the local environs, i.e., that sarcoid is associated with abnormalities of mononuclear phagocytes, in which the mononuclear phagocytes function in an enhanced fashion in their interactions with T-lymphocytes. Consistent with this concept, alveolar macrophages of individuals with sarcoidosis present antigen in an exaggerated fashion to autologous blood and lung T-lymphocytes as well as to HLA-DR-matched T-cell lines. In this regard, it is conceivable that sarcoid results, at least in part, from the heightened response of mononuclear cells to antigens localized at sites of disease. If this hypothesis is correct, it should be possible to identify those genes responsible for the putative hypermacrophagic state. One candidate gene is that coding for interleukin- $1\beta$  (IL- $1\beta$ ), a 17 kDa protein that is the dominant species of interleukin-1 produced by human mononuclear phagocytes. Relevant to the pathogenesis of sarcoidosis, it has been suggested that alveolar macrophages of patients with active sarcoid are spontaneously releasing IL-1, while alveolar macrophages of normal individual are not. If correct, this would help explain why these individuals have exaggerated numbers of activated proliferating T-cells in the lower respiratory tract.





In the context of these considerations, a study was designed to evaluate the expression of the IL-1 $\beta$  gene in alveolar macrophages obtained from the lungs of individuals with active pulmonary sarcoidosis. Evaluation of media from unstimulated cultured sarcoid alveolar macrophages failed to detect IL-1 activity. When parallel cultures of sarcoid and normal alveolar macrophages were stimulated with lipopolysaccharide (LPS), they released similar amounts of IL-1 activity. Using a highly specific polyclonal anti-IL-1 $\beta$  antibody and flow cytometry to evaluate cell-associated IL-1 $\beta$ , analysis of fresh alveolar macrophages from patients with active sarcoidosis and normal individuals revealed no detectable cell-associated IL-1 $\beta$ , but IL-1 $\beta$  was present when macrophages from sarcoid patients and normals were stimulated with LPS. Similar observations were made using immunoblot analysis of cell lysates of the same unstimulated and stimulated macrophages. Finally, Northern analysis of alveolar macrophages for IL-1 $\beta$  mRNA transcripts demonstrated minimal, but equivalent, amounts of IL-1 $\beta$  in both normal and sarcoid macrophages, as compared to the much larger quantities present in LPS-stimulated alveolar macrophages. Thus, while alveolar macrophages of individuals with sarcoidosis are clearly capable of expressing the IL-1 $\beta$  gene, these findings suggest that altered expression of the IL-1 $\beta$  gene by alveolar macrophages does not play a central role in the exaggerated lung T-cell activation characteristic of sarcoidosis.

To evaluate the possible mechanisms underlying the observation that mature populations of human mononuclear phagocytes are relatively poor IL-1 producers compared to blood monocytes, the expression of the IL-1 $\beta$  gene mRNA transcripts was quantified in LPS-stimulated normal autologous blood monocytes and alveolar macrophages using a labeled IL-1 $\beta$  cDNA probe. Although Northern analysis demonstrated that stimulated monocytes and alveolar macrophages both express 1.8 kb IL-1 $\beta$  mRNA transcripts, cytoplasmic dot blot analysis showed that the total IL-1 $\beta$  mRNA content in alveolar macrophages was only 38 $\pm$ 5% of that in blood monocytes after LPS-stimulation. Interestingly, *in situ* hybridization using antisense and sense IL-1 $\beta$  RNA probes revealed that whereas most of stimulated blood monocytes contained IL-1 $\beta$  mRNA transcripts, a significant proportion of autologous alveolar macrophages stimulated in an identical fashion did not express the IL-1 $\beta$  gene. In this regard, within 4 hr, 81 $\pm$ 6% LPS stimulated monocytes contained IL-1 $\beta$  mRNA transcripts. In contrast, 4 hr after stimulation, 16 $\pm$ 9% of alveolar macrophages contained IL-1 $\beta$  transcripts and by 18 hr, this had only increased to 43 $\pm$ 15%. Quantification of the size distribution of the IL-1 $\beta$  mRNA expressing monocytes and alveolar macrophages demonstrated that the majority of monocytes expressing the IL-1 $\beta$  gene were 10 to 15  $\mu$ m whereas the majority of alveolar macrophages were 10 to 25  $\mu$ m i.e., among the population of alveolar macrophages, the cells expressing this gene were not confined to those that were "monocyte-like". Together, these observations suggest that there is a significant heterogeneity among populations of mononuclear phagocytes in their ability to express the gene for IL-1 $\beta$  in response to a standard stimulus, and this heterogeneity explains, in part, the observation that differentiated forms of mononuclear phagocytes such as alveolar macrophages are relatively poor IL-1 producers.

A central question is understanding the pathogenesis of sarcoidosis is to understand why T-lymphocytes accumulate at sites of disease such as the lower respiratory tract. Three general hypotheses can be proposed to



explain the pathogenesis of these T-lymphocyte infiltrations in sarcoidosis. First, the accumulated T-cells may represent a polyclonal T-cell response, perhaps secondary to a generalized enhancement of T-helper cell processes or ineffective T-cell suppressor networks that are not antigen-specific. Second, the T-cells may accumulate secondary to a monoclonal or oligoclonal process such as that observed in malignancies in which a "transformed" cell with a growth advantage accumulates in tissues. Third, the T-cells may accumulate secondary to antigen-driven processes, in which one or several antigens drive the clonal expansion of antigen-specific T-cells together with the secondary expansion of populations of immunoregulatory and/or bystander T-cells. Since the mechanisms responsible for T-cell accumulation in these three categories are different, an understanding of whether the lung T-cells are polyclonal, oligoclonal, or monoclonal is an important step in understanding the pathogenesis of this disorder. As an approach to this question, we have capitalized on the recent identification and cloning of the  $\beta$ -chain gene of the T-cell antigen receptor. Since the  $\beta$ -chain gene of the T-cell antigen receptor undergoes specific DNA rearrangement during normal T-cell ontogeny, these rearrangements serve as a marker for clonal populations of T-cells that can be detected by Southern blot analysis of DNA isolated from T-cells. Using this immunogenotypic approach, we evaluated T-cells from the lung and blood of patients with active sarcoidosis, inactive sarcoidosis and normals. DNA from lung T-cells of 7 of 10 individuals with active sarcoidosis demonstrated non-germline bands on Southern blot analysis using a  $\beta$ -chain gene constant region probe, consistent with the presence of T-cells with "clonal" rearrangements of the  $\beta$ -chain gene locus. In contrast, normal individuals and 5 of 5 cases of inactive pulmonary sarcoidosis had no detectable  $\beta$ -chain gene rearrangements among their lung T-cells. Interestingly, blood T-cell DNA of 6 of 10 individuals with active pulmonary sarcoidosis also demonstrated clonal rearrangements of the  $\beta$ -chain gene, indicating a systemic nature of the T-cell processes associated with this disorder. The  $\beta$ -chain rearrangements of lung and blood T-cells of different individuals with sarcoidosis persisted over many months. Together, these observations suggest that a population of T-cells with a highly restricted repertoire of  $\beta$ -chain gene rearrangements are involved in the chronic inflammatory processes that characterize this disorder.

Current concepts of the pathogenesis of sarcoidosis suggest that the expanded numbers of activated T-helper/inducer cells at sites of disease activity result, at least in part, from their proliferation in the local milieu. Since normal clonal proliferation of T-cells involves activation and expression of the IL-2 receptor and its subsequent interaction with IL-2, we evaluated these concepts by quantifying lung and blood IL-2 receptor gene mRNA transcripts and IL-2 receptor cell surface protein of patients with active and inactive sarcoidosis and normals. Northern analysis of RNA extracted from lung T-cells using a  $^{32}\text{P}$ -labeled IL-2 receptor cDNA probe demonstrated that patients with active sarcoidosis express 3.5 kb and 1.5 kb IL-2 receptor mRNA transcripts, the same as observed in normal T-cells activated in vitro. Consistent with this, using flow cytometry and a monoclonal antibody directed against the IL-2 receptor protein (2A3), significant levels of IL-2 receptor protein were observed on the surface of both lung and blood T-cells of active sarcoidosis patients (lung  $5.7 \pm 2.4\%$ , blood  $5.8 \pm 1.7\%$ ). In contrast, while normal lung T-cells



expressed the IL-2 receptor surface protein ( $2.9 \pm 1.0\%$ ), normal blood T-cells did not contain detectable IL-2 receptor mRNA and IL-2 receptor protein was detectable very rarely ( $0.5 \pm 0.4\%$ ). Thus, sarcoidosis is associated with both tissue and circulating T-cells expressing the IL-2 receptor gene. When placed in the context of the known compartmentalization of T-cell proliferation and spontaneous IL-2 production in active pulmonary sarcoidosis, it is possible that these IL-2 receptor positive cells represent a subset of T-cells that have a proliferative advantage when present in a local milieu with IL-2 present, such as the lower respiratory tract of patients with active pulmonary sarcoidosis.

Pulmonary berylliosis is a chronic granulomatous disorder characterized by accumulation of helper T-cells, macrophages, and granulomas in the lower respiratory tract. To evaluate the hypothesis that the expansion of large numbers of CD4+ T-cells in the lung of patients with pulmonary berylliosis is associated with Be-specific CD4+ T-cell activation, the response to Be of lung T-cells was evaluated in four patients with pulmonary berylliosis. All had abnormal chest X-rays and gallium scans, increased proportions of lung lymphocytes ( $49 \pm 5$ ; normal  $12 \pm 2$ ;  $p < 0.001$ ), increased helper/suppressor ratios ( $7 \pm 4$ , normal  $2 \pm 1$ ,  $p < 0.5$ ) and high proportions of activated (DR+) CD4+ lung T-cells ( $61 \pm 5$  of CD4+, normal  $7 \pm 1\%$ ,  $p < 0.001$ ). To show that the Be-induced T-cell proliferation is HLA-DR restricted, and requires the activation of interleukin-2 receptor (IL2R) pathway, purified lung T-cells + 20% monocytes were cultured with BeSO<sub>4</sub> ( $0.5 \times 10^{-5}$  -  $0.5 \times 10^{-4}$ M), with or without the anti-HLA-DR monoclonal antibody 9.49 or the anti-IL2R antibody anti-Tac, and pulsed with <sup>3</sup>H-thymidine after 5 day culture. T-cell proliferation to BeSO<sub>4</sub> (stimulation index ( $79 \pm 29$ )) was significantly prevented by anti-DR ( $97 \pm 1\%$  inhibition) and by anti-IL2R ( $83 \pm 4\%$  inhibition). To determine which lung T-cell subset proliferated in response to Be, purified lung CD4+ or CD8+ T-cells were cultured with 20% monocytes. While CD4+ lung T-cells proliferated in response to BeSO<sub>4</sub> (stimulation index  $102 \pm 33$ ), CD8+ lung T-cells did not ( $2 \pm 1$ ,  $p < 0.5$  compared to CD4+). In addition, flow cytometry propidium iodide cell cycle analysis of unfractionated lung mononuclear cells cultured with Be for 5 days demonstrated that  $10.8 \pm 2.1\%$  of CD4+ cells were in S,G<sub>2</sub>,M phases of cell cycle compared to  $3.4 \pm 0.9\%$  CD8+ cells ( $p < 0.05$ ). Thus, in individuals with berylliosis, lung T-cells respond to Be with antigen specific, HLA-D-restricted, IL2/IL2R dependent activation of CD4+ T-cells, suggesting this process plays a fundamental role in expanding the helper T-cell population in the lungs in this disorder.

T-lymphocytes on the epithelial surface of the lower respiratory tract are thought to represent a relatively compartmentalized population of T-cells that exchanges slowly with the blood. Since the lung is chronically burdened with antigens, "resident" T-cells likely have a history of past activation. To evaluate this concept, we analyzed resident lung T-cells for VLA-1 expression, which is indicative of a history of past stimulation. Lung lavage and blood T-cells were evaluated in 13 normal nonsmokers using the monoclonal antibodies Leu4 (CD3, pan T-cells), Leu3 (CD4, helper/inducer T-cells), Leu2 (CD8, suppressor/cytotoxic T-cells), TS2/7 ( $\alpha 1$  subunit of VLA-1) and A-1A5 ( $\beta$  subunit of VLA-1) using immunofluorescence and immunoprecipitation. In contrast to normal blood T-cells which did not express VLA-1, lung T-cells expressed the 210 kDa  $\alpha 1$  and 130 kDa  $\beta$  subunits of the



VLA-1 complex, the same as blood T-cells activated in culture for three weeks. Two color immunofluorescence with Leu4 and TS2/7 showed that  $19 \pm 6\%$  of the lung T-cells were VLA-1+, suggesting that a significant proportion of T-lymphocytes on the alveolar epithelial surface are in a separate compartment from the VLA-1 blood cells. In sarcoidosis, a disease characterized by exaggerated numbers of active CD4+ T-cells in the lower respiratory tract, increased numbers of lung CD4+ T-cells expressing VLA-1 were present on the epithelial surface of the lung ( $p < 0.05$  compared to normals). These observations are consistent with compartmentalized, chronically stimulated T-lymphocytes on the alveolar epithelial surface that exchange with the systemic immune system very slowly.

#### Significance to Biomedical Research and the Program of the Institute

The T-lymphocyte disorders of the lower respiratory tract have a prevalence of 25 to 50 per 100,000 in the U.S.A. population. Approximately 50% of these patients have permanent dysfunction of the lung and 10% eventually die from the disorder. The mechanisms of how these T-lymphocytes accumulate in the lung are beginning to be understood. With this understanding, it should be possible to design rational strategies to stage and treat these individuals.

#### Proposed Course

Studies will continue to decipher the mechanisms by which T-lymphocytes accumulate in the lower respiratory tract in each of these disorders and to understand the function of the individual T-lymphocyte subsets in the pathogenesis of the disease states. As these mechanisms are understood, strategies will be developed to attempt to modulate these processes in clinical trials.

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Muller-Quernheim J, Saltini, C., Sondermemer, P., Crystal, R.G.; Compartmentalized activation of the interleukin-2 gene by lung T-lymphocytes in active pulmonary sarcoidosis. Journal of Immunology 1986, 137, 3475-3483.

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ANNUAL REPORT OF THE SURGERY BRANCH  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE  
OCTOBER 1, 1986 THROUGH SEPTEMBER 30, 1987

The clinical and laboratory research performed by the Surgery Branch is focused on elucidating solutions to long-standing clinical problems in cardiac surgery in the following areas: prosthetic heart valves, hypertrophic cardiomyopathy, metabolism during hypothermia with and without ischemia and/or cardiopulmonary bypass, augmentation of blood flow and power for ischemic cardiac failure and rejection phenomena association with cardiac transplantation.

STUDIES OF PROSTHETIC HEART VALVES

Almost 30 years have passed since the first implantation of an artificial heart valve in a human. These devices continue to cause problems of obstruction to flow in small sizes and excess regurgitation in large sizes. There is poor durability ( $\leq 10$  years) for those made from animal tissues. Significant rates of embolic disabilities or deaths continue. Most prosthetic valves require use of anticoagulant therapy which results in a small but constant rate of life-threatening hemorrhage. All valves remain potential sites for lethal endocarditis. Despite the problems, more than one million persons have had implantation of one or more of these devices and more than 100,000 are implanted annually. The research undertaken by the Surgery Branch is directed toward understanding the mechanisms of the deficits and attempting to provide amelioration of the long-term clinical complications.

Five prospective projects and four retrospective clinical studies are in progress or were completed in the reporting interval. M. Jones has concentrated on the evaluation of various processing treatments of animal tissue to retard the mineralization process in vivo of these types of prosthetic heart valves. He has established a juvenile animal system which causes accelerated calcification of bioprosthetic valves: unimplanted valves contain an average of 1 mg of calcium per gram of tissue (dry weight); whereas, 20 weeks after implantation the valves contain 79-190 mg/g. The clinical analog is 1-5 years in children and 3-12 years for adults greater than 35 years old at the time of implant. The experimental data base for the standard porcine aortic and standard bovine pericardial valves, i.e. those now in clinical use is large (161 and 133, respectively).

Against this frame of reference, proposed new treatments to retard the mineralization process were tested. The results show that three treatments reduced mineralization by 92, 81, and 16% compared to standard prosthetic valves made from porcine aortic valves. In contrast, only one of four treatments of bovine pericardial valves reduced calcium content 20 weeks after implantation (30%) and the remaining three actually increased the rate of mineralization by 48, 42, and 59%. Importantly, preliminary data show that treatment with some surfactants, although decreasing calcification, may reduce bioprosthetic tissue durability. A study of mineralization of synthetic trileaflet valves made from Biomer, a linear segmented aromatic ethylene diamine polyether urethane, showed significant calcification in two areas, one



at the blood-material interfaces and the other within thrombi engendered fibrous sheaths.

In all, more than 170 successful implants were obtained in this reporting interval, making this endeavor the world's foremost.

Ultrasonic technologies assessment have been used in 120 acute, short-term (1-14 days) or long-term ( $> 20$  weeks) animal valve implant studies. Additionally, ten new studies (total = 20) of normal flow patterns through the left atrium, mitral valve and left ventricular outflow tract were performed and ten similar studies were performed after mitral ring annuloplasty. The results of this program using M and 2-D imaging modes, and pulsed, continuous and color-encoded Doppler have resulted in the following major accomplishments:

- 1) The normal patterns of blood flow within the left ventricle and the mechanism of mitral valve closure have been characterized in vivo for the first time.
- 2) The importance of orientation of eccentric orifices of tilting disc mechanical prosthetic valves has been determined. Orientation influences the velocity pattern of blood within the left ventricle. One orientation results in a uniform flow toward the apex, septum and left ventricular outflow tract. Orientation change of  $180^{\circ}$  results in blood flowing along the septum and free wall such that flow is reversed in the minor orifices outflow region of the prostheses while blood continues to flow antegrade through the major orifices. These phenomena could be responsible for the formation of thrombi in the minor orifice, result in embolic events, and/or cause hemolysis.
- 3) The flow patterns of all types of prosthetic heart valves have been characterized at various heart rates, flows, and preload and afterload conditions. The ultrasonic data have been compared to those obtained by laser Doppler anemometry. The data are highly concordant qualitatively and are in good quantitative agreement.
- 4) The limitations of these various ultrasonic systems to accurately quantitate regurgitant velocities through surgically created orifices in the anterior leaflet of the mitral valve have been described. The important conclusion from these studies was that both devices tend to "over read" or over estimate, hence should not be used as the sole clinical criteria for decision making with respect to need for operative intervention.

Additionally, in vitro hydraulic, hemodynamic, ultrasonic, laser light, flow visualization photographic studies have been conducted in two locations. The four year collaboration with Dr. Yoganathan at the Georgia Institute of Technology has continued. During the reporting interval, the previously purchased in vitro testing equipment became fully operational in house with the significant help of BEIB in developing a computer interface system for microsecond data acquisition and controlling function of the photographic apparatus. Two sizes each of 6 mechanical heart valves of three



types were studied using sheep blood. Four cycle rates were used. The maximal volume and velocities were measured ultrasonically and phasic pressure differences across prostheses were measured manometrically. The regurgitation during and after closure were measured simultaneously by continuous wave Doppler and electromagnetic methods. Flow visualization studies used color-encoded Doppler and computer controlled photographic cameras and laser light.

The results show that the maximal pressure differences were equally determined by both methods ( $r > 0.95$ ) and were a function of the distance measured from the valve ring housing. Additionally, the ultrasonic color-encoded velocity patterns were highly concordant with those obtained by laser illuminated amber light particle photographic and video studies. These data provide a frame of reference for measurements to be made on new bioprosthetic valves, degenerated valves removed from sheep and patients and prototype valves.

Studies continued on the effect of limited or no resection of the mitral valve apparatus during mitral valve replacement in patients with mitral regurgitation. No deaths or complications have resulted and significant low cardiac outputs have not been observed. The hypothesis tested in this clinical study and the ongoing animal studies is that preservation of leaflet-chordal-papillary muscle continuity of the mitral valve prevents or ameliorates myocardial dysfunction commonly seen immediately after mitral valve replacement (MVR) in the dilated left ventricle. Chronic long-term animals with surgically created mitral regurgitation were used. The data show that in normal animals, MVR and chordal transection result in minimal alteration of left ventricular geometry and small changes in systolic and diastolic contractility and compliance. In contrast, MVR in the presence of significant chronic mitral regurgitation, results in major decrements in systolic function and compliance if discontinuity is created in the mitral valvular apparatus during mitral valve replacement. These data, soon to be submitted for publication, are the basis for the continued clinical trial.

Two major retrospective studies of the clinical results of prosthetic valve replacement therapy have been published or are in press. These demonstrated that the long-term results of aortic valve replacement with very small devices did not result in poor results as previously thought. Second, the use of a porcine aortic valve mounted on a cloth covered stent implanted in the mitral position gave satisfactory results for five years. Structural deterioration became evident at 8 years (20%) and was highly significant at 11 years (40%). Two other studies concerning the long-term complications of bioprosthetic mitral valve replacement and mitral valve replacement in children have been completed and manuscripts have been prepared.

#### OPERATIVE PALLIATION OF IDIOPATHIC HYPERTROPHIC SUBAORTIC STENOSIS

463 patients have had the operation devised by A.G. Morrow, left ventricular myotomy and myectomy (LVM&M). This operation is a partial resection of the left side of the ventricular septum based upon intraoperative ECHO findings. 330 patients were operated upon by Morrow and 133 patients by C.L. McIntosh, M.D.. Intraoperative echo cardiographic studies are used to determine the appropriateness of this procedure or mitral valve replacement.



(infra vide). Data analyses have been performed for each operative subset, the resting and provokable left ventricular outflow pressure gradients before and after the procedure, the presence or absence of coronary artery disease, and the presence of other major cardiac abnormalities - e.g. aortic or mitral valve dysfunction or disease, right ventricular manifestation of IHSS etc. The data demonstrate that when no co-existing cardiac disease existed and only the standard operation (LVMM) was performed, of 133 treated patients, 4 (3%) have died acutely ( $\leq 30d$ ). Total perioperative mortality was strongly related to the need for coronary artery bypass grafting, and operative treatment of other complex intracardiac disease. Resting and provokable pressure gradients have been reliably relieved. The degree of relief of obstruction has no relation to the degree of symptomatic relief experienced by these patients which is often dramatic. Control of the arrhythmic component of this disease continues to be the most difficult aspect of aftercare. These data suggest that the combination of improved myocardial protection and drug therapies now available are responsible for a decrease in operative mortality.

The prospective study of use of mitral valve replacement (MVR) for patients with IHSS not thought to be candidates for the classic procedure has been in progress for the past four years. The indications for this operation are: 1) thickness in the basal portion of septum of  $\leq 18$  mm; 2) atypical septal morphology, 3) persistent obstruction after a previous septal resection, 4) those with severe mitral regurgitation secondary to organic valve disease. Fifty-eight have had MVR of whom 4 (6.9%) have died in the perioperative interval. Thirty-six patients have been restudied and the data demonstrate relief of the outflow tract obstruction which is equal to that achieved at rest with the classic procedure (LVMM). These data are to be presented and published in the near future and represent a major contribution in the treatment of this disease. The Morrow procedure requires specialized training to perform this operation safely and is currently only performed in volume at NIH. Mitral valve replacement is a common procedure and can be performed by all well-trained cardiac surgeons throughout the world.

The retrospective studies were completed and accepted for publication. The first analyzed the data for patients with IHSS who had the Morrow procedure and were over 65 years of age. The results showed that if there was no co-existing cardiac disease that the perioperative mortality rate was 8%, an acceptable value by historical standards. The five year survivorship was 82%. In the presence of coronary disease, the mortality rate doubled. These data were analyzed from pre 1981 data base, hence the improvements now existing were not evident.

The second study considered the role of relief of obstruction by LVMM in the mechanism of mitral regurgitation, commonly found in operative patients with IHSS. The data demonstrated that only one half of the patients had an improvement in mitral regurgitation and was unrelated to postoperative hemodynamic data or symptomatic relief. Hence, the conclusion was made that left ventricular obstruction is a contributing augmenting factor to mitral regurgitation but not the genesis which is probably the congenital abnormal geometric relation of the mitral valvular apparatus within the left ventricle.

The third study reported the influence of the pre-operative use of the antiarrhythmic drug amiodarone on outcome after operations for IHSS. The data from 16 patients so treated showed that there was a major increase in the





incidence of death and life threatening complications of cardiac, pulmonary and hepatic dysfunction especially in those treated with more than 100 gms of drug which was equivalent to 9 to 12 months of therapy.

### METABOLIC STUDIES

This area of investigation is not new to the Branch from a historical perspective but the formalization of effort in selected areas is the result of the efforts of Dr. J.A. Swain. Central to these investigations is the present lack of knowledge of hydrogen ion flux and control during hypothermia. Various studies by physiologists have demonstrated apparent differing behavior of acid-base regulation in ectotherms and hibernating animals. Two recent technologies have opened new areas of experimentation: nuclear magnetic resonance spectroscopy and fiberoptic tissue pH transducers. Animal and human studies are in progress. The 4.7 Tesla NMR unit has been used to a) determine myocardial energy state as a function of various metabolic states, and b) determine myocardial and cerebral pH alterations associated with hypothermia. These studies have been complemented by determination of acid-base changes in dogs and sheep during increments of hypothermia and the consequences of using various schemes to control hydrogen ion concentration during the graded changes of temperature. The clinical studies have been performed on patients undergoing cardiac surgery who necessarily require use of the heart-lung machine, hypothermia, and steady state flow conditions for periods of one to three hours. Myocardial and perfusate pH are continuously measured. Acid-base regulation is randomized to two modes; control by carbon dioxide concentration in the blood oxygenator such that pH of the perfusate remains at  $7.40 \pm 0.5$  measured at  $37^{\circ}\text{C}$  or remains at 7.40 at the temperature of the perfusate. The data to date demonstrate that dogs are not suitable subjects for measurement of myocardial pH by NMR because of the similar resonance frequencies of inorganic phosphate from which the intracellular pH is derived mathematically and 2,3-DPG in the blood. The preliminary data from sheep, which lack 2,3-DPG, have demonstrated the feasibility of continuation of these studies. The pH data of human myocardium during increasing hypothermic levels without global ischemia in 14 patients has shown that cardiac pH follows that of the blood perfusate. Hence, the regulation of hydrogen ion concentration in the perfusate is the fundamental variable in humans, which is unlike that which occurs in hibernators. The method resulting in the least alteration of function appears to be the one that increases pH as temperature decreases and parallels the change in the dissociation constant of water.

The data for 30 patients who had hypothermic cardiopulmonary bypass (CPBP) and had metabolic measurements made through the operative course and for the first six hours in the intensive care unit showed significantly less acidosis during CPBP and less rebound alkalosis during recovery in those patients who had blood pH kept at 7.40 (as measured at  $37^{\circ}\text{C}$ ). These data strongly suggest that organ function can be better preserved by employing an acid-base regulation method which follows that of ectotherms. Further, these clinical data are supported by 18 studies in dogs which demonstrated significant lactic acid production indicative of anaerobic metabolism in those animals ( $n=13$ ) in which the normothermic pH was held constant at various hypothermic levels although systemic oxygen consumption levels were equal. These data suggest that hydrogen ion management has a profound effect on



enzymatic activity and oxygen utilization during hypothermic states.

A clinical study has been initiated to investigate the role of the type of fluids used during hypothermic cardiopulmonary bypass on the changes of interstitial lung water. The measurement of extravascular lung water uses the simultaneous double indicator colorimetric method. Patient subsets are those with and without left heart failure and those with and without pulmonary hypertension. Two fluid management methods are used: crystalloid only or colloid and crystalloid. The preliminary data demonstrate a wide variance in extravascular water of cardiac patients e.g. 500-800 ml. Further refinement of the experimental measurement technique has reduced these variations. This study is to continue.

#### AUGMENTATION OF END STAGE ISCHEMIA AND CARDIAC FAILURE

Previous studies from this Branch have demonstrated that normal dogs can be maintained long-term in apparent good health with the only blood supply to the left ventricle being two implanted internal mammary arteries, the native circulation having been occluded gradually by ameroid constrictors. Microsphere data demonstrated only a small increase in myocardial blood flow with isoproterenol infusion with mild alterations in distribution between the endo and epicardial layers. The internal mammary arteries develop a network of small arterioles which interconnected to the existing mid-myocardial vascular network. These data suggest that the new network could be enhanced by accelerating the native angiogenic process. These studies stimulated a series of investigations on the aciditic fraction of an endothelial cell growth factor derived from brain and now available from genetic engineering sources. This material strongly binds heparin and by doing so increases the half life of the biologic activity 5-10 fold. The new nomenclature for this protein is heparin binding growth factor I (HBGF-I). A series of studies were undertaken to understand the mechanisms involved. First, it was shown that heparin markedly inhibited proteolytic degradation to a host of proteases and resulted in retardation of thermal denaturation. Additional studies used human endothelial cells and growth activity was greatly enhanced with the combination of heparin and HBGF-I. Subsequent studies demonstrated that heparan sulfate and other low molecular weight non-anticoagulant compounds produced equal biologic effects. Next, the pharmacokinetics and distribution of HBGF-I were studied in the rat. The compound binded to all organs equally except the kidney. Saturation occurred at 10 ng/ml serum concentration. The endothelium of the rat carotid artery was denuded by a balloon abrasion method and HBGF-I with and without heparin fragment was used. Heparin fragments alone increased endothelial regeneration probably by the mechanism of binding with the native endothelial cell proteolytic enzymes and extending the half life of native endothelial cell growth factor. The combination of exogenous HBGF-I and heparin further augmented regeneration. These studies suggest that it is possible to stimulate endothelial regeneration in areas of injury and accelerate the process of angiogenesis. Other studies conducted on mammalian hearts of various species demonstrated a heretofore unknown compound, a third acid-heparin binding growth factor which was designated, gamma. It was sequenced and found to fall midway between the previously known alpha and beta moieties. These studies led to the study of young, mature, and aging human endothelial cells grown in culture and the response to heparin fragments by study of gene expression. It was shown that these three general types of cells had different expressions and that aged cells could be altered in the



presence of heparin and HBCF-I to act in a manner similar to mature cells. These data have implications with respect to the mechanisms involved in atherosclerosis and potential usefulness in cardiac and vascular surgery. Other human endothelial cell studies were performed with a parallel flow plate apparatus to study responses to steady state and pulsatile flow within the apparatus. Cell shape, alignment and gene expression were markedly altered in a pulsatile flow field. These data suggest that kinetic energy strongly influences metabolic activity of endothelial cells. These data will be used in large animals to study the feasibility of augmentation of blood supply to chronic ischemia hearts by employing heparin fragments and HBGF-I infusions, autologous endothelial cells implantation and augmentation with arterial implants and organ overlay procedures. The power augmentation program has only recently been initiated to improve right ventricular dysfunction and left ventricular function with aortic wraps of vascularized skeletal muscle pedicles. These studies are directed toward palliation of those patients with ischemia cardiomyopathies and congestive heart failure who are no longer suitable candidates for conventional therapies.

#### MECHANISMS OF ORGAN REJECTION

One study in modification of organ rejection was undertaken during the reporting interval. A monoclonal anti-Tac antibody was developed for the Rhesus monkey by the Immunology Branch of NCI. This antibody recognizes the IL-2 receptor of activated T-cells, a cell which plays a major role in acute and chronic rejection. Cynomolgus monkey hearts were transplanted into the necks of Rhesus and the anti-Tac antibody alone, anti-Tac chelated with pseudomonas exotoxin and with <sup>90</sup>-Yttrium were given. Anti-Tac alone had no effect. The exotoxin conjugate was highly toxic. The beta-emitter conjugate prolonged rejection from 6 to 32 days although the irradiation effects had a strong detrimental effect on both white and red cell production. Efforts are to continue to determine the effect in allografts with and without cyclosporine A to determine if less toxic immunosuppressive regimens can be devised.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02714-07 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evaluation of Prosthetic Cardiac Valve Failure in an Animal Model Study

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI

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Stephen L. Hilbert, Ph.D., Center for the Devices and Radiological Health, FDA

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Pathology Branch, NHLBI

Center for Devices and Radiological Health, Food and Drug Administration

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

10

## PROFESSIONAL:

5

## OTHER:

5

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The purpose of this project is to develop an animal model to evaluate cardiac valves in vivo. This animal model system is utilized in a multidisciplinary manner, including the fields of surgery, physiology, pathology, engineering and technology development. We have shown that bioprosthetic valves implanted in juvenile sheep demonstrate the same pathologic alterations of degeneration and calcification as those implanted in humans; however, the development of these alterations is accelerated in sheep as compared to humans. Nearly 600 porcine aortic or bovine pericardial bioprosthetic valves from fourteen sources and of more than thirty different types have been implanted in the animal model system to: 1) assess the characteristics of the pathologic changes, 2) compare the alterations in different types of valves, 3) compare the alterations in different types of valves, 4) compare the alterations occurring in valves implanted in the mitral versus the tricuspid positions, and 4) evaluate valves treated prior to implantation with processes to retard or to eliminate the calcification and degeneration processes. Due to these types of observations, clinical trials have been initiated with two new types of bioprosthetic valves. Equally important, if not more so, at least seven types of valves have been rejected from consideration for clinical use. Both the positive and negative results of studies of these processes have added insight to the mechanisms of calcification, degeneration and malfunction of prosthetic cardiac valves. Additionally, the model is being utilized to validate ultrasonic techniques, including color-encoded, two-dimensional Doppler, for the characterization of bioprosthetic, mechanical, and synthetic leaflet valve velocity flow turbulence profiles and for the noninvasive detection of valve failure.

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Project Description: Although more than 20 years have passed since the first prosthetic cardiac valves were implanted in humans, the development of an ideal cardiac valve substitute remains a major problem in cardiac surgery. Bioprosthetic cardiac valves have become the valves of choice at many institutions and for certain subgroups of patients, primarily because they do not require chronic anticoagulant therapy. However, it is now apparent that the long-term durability of bioprosthetic cardiac valves is finite; degeneration and calcification of these valves are the major complications of long-term implantation. It is estimated that more than one-half million bioprosthetic valves have been implanted in human patients to date. Of additional concern is the fact that somewhere between 50-100,000 valve replacement operations continue to be performed annually world wide.

An in vivo, in situ investigational model is essential for the study of the mechanisms of degeneration and calcification in substitute bioprosthetic cardiac valves. For this purpose we have met the following criteria in an animal model system developed in domestic sheep (Ovis aries): (1) the bioprostheses should be implanted in growing animals to simulate the physiologic conditions in young humans in whom accelerated degenerative alterations of bioprosthetic valves are known to occur; (2) the pathologic alterations should develop within several months after implantation to permit expeditious study; (3) at maturity the animals should not outgrow the bioprosthetic cardiac valves; (4) the animals must be of suitable sizes for standard cardiopulmonary bypass techniques; and (5) the pathologic alterations in the bioprosthetic valves must be similar in the animals and in humans.

During the past four years an animal model system has been developed in juvenile (10-15 week old) sheep which permits early and late studies of host-prosthesis interactions. Over 600 porcine aortic valves (PAV) and bovine pericardial valves (BPV) have been implanted in the tricuspid (TVR) or mitral (MVR) positions. In analyzing the results obtained with valve implantation in this model system, emphasis has been given to: (1) early and late hemodynamic investigations of valve function; (2) pathologic alterations, including localization of calcific deposits; (3) quantitative analyses of valve calcification; (4) evaluations of treatment processes to decrease valve calcification; and (5) use of intracardiac Doppler 2-D echocardiography to study valve function. Early hemodynamic studies showed anticipated performance for the manufacturer's size 25 mm valves utilized:

	Mean Gradient (mm Hg)	Gorlin Valve Area (cm <sup>2</sup> )
TVR - BPV	4.4 ± 0.3 (n = 61)	1.98 ± 0.16 (n = 61)
TVR - PAV	5.4 ± 0.3 (n = 88)	1.61 ± 0.11 (n = 88)
MVR - BPV	7.4 ± 0.2 (n = 193)	2.02 ± 0.06 (n = 174)
MVR - PAV	8.2 ± 0.2 (n = 256)	1.74 ± 0.05 (n = 252)

Cardiac outputs = 3.01 ± 0.07 l/min (n = 667). At the time of explantation (average 5 months) hemodynamic observations reflected pathologic alterations. Pathologic changes resembled those found in humans, including perforations; microemboli; red blood cell, round cell and giant cell infiltrations; fibrous sheathing with and without cuspal retraction and commissural fusion; insudation of plasma proteins; cuspal delamination; infection; presence of cotton fragments; strut creep; cuspal abrasion and perforation by sutures; and calcific deposits. Morphologic sites of calcific deposits included cuspal connective tissue and cuspal cells, the muscle shelf and aortic wall of PAV's as well as microthrombi, vegetations and fibrous sheaths. Cartilage and bone



formation have also been observed.

Whereas, nonimplanted valves contained  $0.78 \pm 0.09$  mg of calcium per g of tissue dry weight ( $n = 45$ ), the following results were obtained for standard porcine aortic (PAV) and bovine pericardial (BPV) valves implanted in the mitral (MVR) or tricuspid (TVR) positions:

	<u>N</u>	<u>Calcium content (mg/g)</u>		<u>N</u>	<u>Calcium content</u>
PAV-TV	44	$84.6 \pm 10.9$	PAV-MV	117	$91.8 \pm 5.5$
BPV-TV	27	$190.4 \pm 49.1$	BPV-MV	106	$79.3 \pm 5.0$

A major continuing emphasis of these studies during the past year has been studies of the efficacy of various preimplantation treatment regimens upon ameliorating the calcification of bioprosthetic valves. Comparisons have been made of low versus high pressure glutaraldehyde fixation of the bioprosthetic valve. Studies performed by other investigators using the subcutaneous implantation of valvular tissue in the rat model have indicated that a number of preimplantation of valvular tissue in the rat model have indicated that a number of preimplantation treatment of processes inhibited calcification. Therefore, using the sheep model system we have, and are currently, evaluating the following treatment processes: 1) the use of surfactants, i.e., sodium dodecylsulfate, polysorbate 80, Triton X-100, or N-lauryl sarcosine; 2) the use of diphosphonates (ethanehydroxydiphosphonate or covalently bound aminohydroxypropane diphosphonic acid); 3) the use of toluidine blue; 4) the use of a prostaglandin/prostacyclin inhibitor; and 5) the incorporation of a polyacrylamide. Results obtained thus far are given in the table below.

CALCIUM CONTENT OF BIOPROSTHETIC  
VALVES IMPLANTED IN THE MITRAL POSITION

<u>Valve Type</u>	<u>n</u>	<u>Calcium Content (mg/g tissue)</u> 20 wks after implantation
Standard porcine aortic	117	$91.8 \pm 5.5$
Polysorbate-80/Mg/Hepes treated aortic	15	$7.6 \pm 2.6$
Sodium dodecylsulfate treated aortic	17	$17.7 \pm 4.2$
Toluidine blue treated aortic	25	$77.3 \pm 10.9$
Standard bovine pericardial	106	$79.3 \pm 5.0$
Polysorbate/Mg/Hepes treated pericardial	11	$55.2 \pm 12.7$
Sodium dodecylsulfate treated pericardial	24	$117.7 \pm 5.3$
Polyacrylamide treated pericardial	8	$112.9 \pm 15.3$
Aminohydroxypropane diphosphonate treated pericardial	10	$126.3 \pm 8.7$

Data = mean values  $\pm$  S.E.M.



Thus, of the studies completed at the present time only the two types of surfactant treatments (Polysorbate-80 and sodium dodecylsulfate) appeared to mitigate significantly the calcification, and only in the porcine aortic valves. However, preliminary studies with other surfactants indicate that these types of treatments may be detrimental to bioprosthetic valvular tissues in terms of durability. Studies of the other types of treatments are ongoing.

Morphologic and hemodynamic studies were made of 8 prototype polyurethane trileaflet cardiac valve prostheses that have been implanted in the mitral position. The leaflets were fabricated of linear segmented aromatic ethylene diamine chain extended polyether urethane (Biomer). Twenty weeks after implantation, calcification of the polyurethane leaflet surfaces was the principle finding. Two distinct types of calcification were observed: one was associated with the polyurethane surface or the interface between the leaflet surface and microthrombi or fibrous sheaths; the other was characterized by calcification associated with degenerated cells within thrombotic material and the fibrous sheath. The results of this study indicated with prosthetic valves with leaflets fabricated of Biomer polyurethane calcify to a clinically important degree, comprising both the hemodynamic performance and biomaterials durability of this type of prosthetic valve.

Thus, implanting bioprosthetic valves in young sheep provides an excellent in vivo, in situ means for investigating prosthetic valves' preparation and design.

Proposed Course: On-going studies include comparisons of the various types of valves, comparisons of the alterations occurring in the mitral versus the tricuspid positions, comparisons of new generation versus old generation valves, comparisons of in vitro versus in vivo performance, evaluations of methods to ameliorate or eliminate the degenerative processes, and validation of ultrasonic techniques of evaluating pathophysiologic abnormalities produced by the valves.

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Abstracts Published:

Hilbert SL, Ferrans VJ, Yoganathan AP, Jones M: The use of buffered saline - benzyl alcohol as a storage solution for explanted bioprosthetic valves implanted in sheep. J Thorac Cardiovasc Surg. (in press).

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02731-05 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Operative Assessment and Results of Left Ventriculomyotomy and Myectomy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

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## COOPERATING UNITS (if any)

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## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A standard left ventriculomyotomy and myectomy (LVMM) has been performed for relief of left ventricular outflow tract obstruction secondary to idiopathic hypertrophic subaortic stenosis (IHSS) in 463 patients. This report summarizes 133 patients undergoing an LVM&M since January 1982. An attempt has been made to define criteria for choice of operation, LVM&M vs. mitral valve replacement (MVR) based upon septal thickness, distribution of hypertrophy, level of systolic anterior motion (SAM), contact of septum, and concomitant coronary artery disease. Intraoperative 2-D and M-mode echos have been performed on a number of these patients providing precise data utilized intraoperatively. Patients with concomitant CAD are at greater risk for an iatrogenic VSD creation which may be avoided by a modified LVM&M or MVR. Operative mortality is 3.0% and late mortality 3.0%. Results are presented based on preoperative resting gradients < 50 mm Hg and > 50 mm Hg. Postoperative hemodynamic studies reveal good relief of resting gradient in most patients but significant provokable gradients remain in some patients. Three patients have demonstrated significant RVOT obstruction (> 50 mm Hg) and underwent concomitant LVM&M and resection of RVOT obstruction. Two patients developed late VSD's; one required closure for a QP:QS of 2.6:1. Reoperation has been performed in some patients with persistent symptoms and gradients. Medical therapy is continued in patients with significant gradients regardless of symptomatic status.



Project Description: Four-hundred sixty-three patients have undergone operative palliation for resting or provokable left ventricular outflow tract (LVOT) obstruction secondary to idiopathic hypertrophic subaortic stenosis (IHSS). All patients reported had the classic Morrow operation (left ventriculomyotomy and myectomy LVM&M) and the last 133 patients will be summarized. Mitral valve replacements has been performed in an additional 58 patients for treatment of their IHSS. Choice of operative approach is based upon intraoperative echocardiographic examination of the septum regarding septal thickness, distribution of septal hypertrophy; i.e., localized or homogeneous and contact point on septum of the anterior leaflet. The preoperative M-mode and 2-dimensional echocardiograms may vary depending upon body habitus providing less precise morphology. A post resection echocardiogram is also performed which has been helpful in predicting relief of LVOT obstruction. Indications for MVR rather than LVM&M include: (1) septum  $< 18$  mm in thickness; (2) unusual septal morphology, i.e., prominent Seipp's notch or hypertrophy located out of the "operative window"; (3) previous LVM&M with persistent symptoms and gradients; (4) severe mitral regurgitation. The LVM&M is performed as described by Dr. Morrow, but myocardial preservation is now accomplished with cold ( $4^{\circ}\text{C}$ ) cardioplegia, topical hypothermia with iced slush and  $26^{\circ}\text{C}$  total body hypothermia.

Patients were placed in two groups based on preoperative hemodynamic data. Group I includes patients with  $\geq 50$  mm Hg resting gradients and Group II includes patients with  $\leq 50$  mm Hg resting gradients, who are generally recommended for operation because of significant provokable gradients.

Group I - Patients with  $> 50$  mm Hg Resting Gradients  $\pm$  CAD: One hundred seven patients, 56 males (mean age 41.7 yrs) and 51 females (mean age 57.4) having resting gradients  $\geq 50$  mm Hg have undergone an LVM&M for relief of LVOT obstruction secondary to IHSS. All were Functional Class III-IV prior to operation. There were 8 early deaths ( $< 30$  days: 7.5%) and 3 late deaths ( $> 30$  days: 3.0%). Early deaths were attributed to low cardiac output ( $n=5$ ); iatrogenic VSD and low cardiac output ( $n=1$ ); iatronic VSD requiring reoperation leading to death ( $n=1$ ). There were a total of 4 VSD's in this group; all had concomitant CAD. Thirteen of 107 patients (12%) had concomitant CAD. Other operations performed included aortic valve replacement ( $n=5$ ); AVR + CABG ( $n=1$ ); tricuspid valve replacement ( $n=1$ ); closure of iatrogenic VSD early ( $n=2$ ); late ( $n=2$ ); AVR, MVR (1); and resection right ventricular obstruction ( $n=3$ ). Postoperative hemodynamic studies continue to show excellent relief of resting gradients with persistent significant provokable gradients demonstrated in some patients. Clinical improvement does not appear to be dependent upon relief of provokable gradients. Three patients were found to have significant right ventricular obstruction ( $> 50$  mm Hg) and at operation a large moderator band was resected in one and the crista supraventriculars plus moderator band in the other. These three patients are the first with biventricular obstruction to undergo operative repair. One patient developed a late VSD with demonstrated QP:QS=1.1:1 at catheterization.

One patient died postoperatively secondary to low cardiac output. The other two patients demonstrated moderate pulmonary hypertension when studied postoperatively.

Group II - Patients with  $< 50$  mm Hg Resting Gradients  $\pm$  CAD: Thirty, 21 males (mean age  $41.7 \pm 7.8$ ) and 9 females (mean age  $58.4 \pm 7.8$ ) were found to have



resting gradients  $\leq 50$  mm Hg and underwent LVM&M. Six patients (20%) had concomitant CABG. There was one early death (4.0%) secondary to persistent bleeding and hypotension in a patient who had undergone a previous CAD and one late death (4.0%) attributed to congestive heart failure in a patient with a dilated cardiomyopathy with only a provokable gradient. Morbidity included 4 iatrogenic VSD's (2 patients had CAD), 3 closed at initial operation (1 patient died) and one occurring late which was repaired seven days after LVM&M with survival. One patient required a pacemaker for complete heart block; 1 patient had a preop AV sequential pacemaker placed for pre-existing RBBB and posterior L hemiblock; 1 patient developed mediastinitis; 1 patient developed a compartment syndrome of leg with no apparent etiology. Hemodynamic studies reveal significant reduction in resting and provokable gradients as previously reported. Thus, LVM&M continues to provide excellent clinical and hemodynamic results in patients with LVOT secondary to IHSS. Using exclusion criteria noted for performing an LVM&M only one patient developed a late VSD. Patients with persistent significant provokable gradients or who are symptomatic will continue to be treated with beta blockers or calcium channel blockers. Long term follow-up will be necessary to determine if the attrition rate of 3-4% in the IHSS population who have not been operated upon will be changed by LVM&M with or without medical therapy.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02733-04 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mitral Valve Replacement in Selected Patients Having IHSS

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

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## COOPERATING UNITS (if any)

Cardiology Branch

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mitral valve replacement (MVR) has been performed on 58 patients as primary or secondary treatment of severely symptomatic patients with resting and/or provokable pressure gradients across the left ventricular outflow tract (LVOT) secondary to idiopathic hypertrophic subaortic stenosis (IHSS). Indications for MVR include: 1) septal thickness < 18 mm; 2) persistent LVOT obstruction after a prior adequate left ventriculomyotomy and myectomy (LVM&M); 3) atypical septal morphology; and 4) severe mitral regurgitation secondary to ruptured chordae tendinae or papillary muscle. Intraoperative echocardiography has provided definition of septal morphology allowing selection for MVR. There have been 4 (6.9%) perioperative deaths: one a result of hepatic failure, one suspected to be caused by prosthetic valve malfunction, and two secondary to infection. Three patients (6.9%) died after hospital discharge, two suddenly and one of congestive heart and respiratory failure. One patient had a late central embolus. Symptomatic improvement to NYHA functional class I or II has occurred in 86% of 36 patients returning for postoperative evaluation. Excellent relief of both resting and provokable gradients has been demonstrated. Four patients continue to be symptomatic (FC III) and have been shown to have abnormal coronary vascular resistances with no reserve indicating the presence of severe small vessel disease. Thus, relief of LVOT obstruction does not always relieve symptoms of chest pain and fatigue. Long-term follow-up will be necessary to assess late mortality and morbidity which will be compared to the well-known results of LVM&M used for palliation in IHSS for the past 26 years.





Project Description: Left ventriculomyotomy and myectomy (LVMM) has become the procedure of choice in patients having LVOT obstruction secondary to IHSS in this clinic. This procedure has been utilized as primary treatment in 463 patients and long-term functional and hemodynamic results have been well established over 25 years. The age of our adult population recommended for operation is increasing secondary to the variety of beta blockers and calcium channel blockers currently available for treatment of IHSS. Intraoperative echocardiography has provided an increasing appreciation of the spectrum of septal morphology that occurs in this disease and has provided the rationale and selection of patients to be palliated with an MVR rather than the standard LVM&M. Current indications for MVR as primary operative treatment are: 1) septum 18 mm or < in area of resection (n=29 patients); 2) atypical septal morphology (n=10); 3) mitral regurgitation related to endocarditis or ruptured chordae tendineae or papillary muscle secondary to ischemic disease (n=5); and secondary treatment for persistent obstruction following an adequate LVM&M (n=12).

Sex and age distribution of these 58 patients is 23 males, mean age 51 years (range 29-71) and 35 females, mean age 49.6 years (range 15-78). Thirty-six patients have returned for postoperative clinical and hemodynamic study approximately 6 months after operation. Preoperative function class (New York Heart Association) was III for 26 patients and IV for 10 patients. Mitral valve replacement was performed using standard extracorporeal techniques and cold cardioplegia for myocardial protection. The Bjork-Shiley spherical disc prosthesis was used in most patients unless there was a contraindication to anticoagulants; then a Hancock bioprosthesis was selected. Because of the prominent left ventricular hypertrophy (LVH) and normal mitral valve annulus size, smaller prostheses (23-27 mm) were implanted. Five of 58 patients required concomitant CABG in addition to MVR. There were 4 (6.9%) early deaths (< 30 days) one secondary to hepatic and renal failure of unexplained etiology, one suspected prosthetic malfunction, and two of infection.

Postoperative functional class in the 36 patients evaluated was I in 15 patients; II in 16 patients, 4 patients in class III, and 1 patient class IV. Therefore, 86% of patients had improved to functional class I or II status following MVR. Four patients undergoing MVR after a LVM&M continue to be functional class III and coronary sinus flow studies have indicated limited coronary flow implicating small vessel disease as probable etiology of continued symptoms. There has been one central embolus, but no anticoagulation complications in returning patients. A perivalvular leak occurred in 1 patient and required repeat operation for closure. One early operative death may have been related to prosthetic malfunction and suspected in two late sudden deaths occurring 4 and 8 months after operation.

Hemodynamic results have been excellent as shown in previous reports. MVR has provided excellent relief of resting and provokable gradients. In spite of the smaller sizes of prostheses implanted, significant prosthetic stenosis has not been demonstrated and pulmonary artery wedge pressures remain in high normal range. MVR is indicated in patients having septums 18 mm or less to avoid an iatrogenic VSD and in previous patients having had an LVM&M with persistent gradients.

Proposed course: Long-term follow-up will be necessary to establish survival, particularly deaths which may be prosthesis related and long-term symptomatic



relief. Extreme left ventricular hypertrophy may distort the mitral valve annulus causing intermittent sticking of the disc and prosthetic malfunction as suspected in several patients in this series. Survival curves will also be derived concerning complications such as emboli, anticoagulation complications and prosthetic malfunction requiring reoperation for comparison to similar curves for patients undergoing LVM&M. The significance of small vessel disease in these patients must be determined.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02740-04 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Coronary Vascular Resistance and Cardiac Metabolism in the Perioperative Period

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

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## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Coronary artery spasm has been implicated in perioperative cardiac arrest, hemodynamic collapse, and myocardial infarction. It is possible that other perioperative events such as low cardiac output, myocardial irritability, and early coronary artery bypass graft closure may at times be due in part, or entirely to, increased coronary vascular resistance (CVR) secondary to effects of circulating catecholamines, intraoperative cardioplegia, hypothermia, anesthesia, or drug effect. Patients who undergo coronary artery bypass have a different endocrinologic response to operation than valve patients, especially when comparing vasopressin levels and the degree of postoperative hypertension, which may affect coronary vascular resistance and the incidence of coronary spasm.

By using a thermodilution catheter placed in the great cardiac vein via the coronary sinus to determine coronary blood flow, it is possible to measure changes in CVR and lactate metabolism in the perioperative period. Reductions in coronary blood flow and alternations in cardiac metabolism during coronary spasm have been documented using a coronary sinus catheter.

The purpose of this study is to compare changes in coronary blood flow and resistance, lactate metabolism, and oxygen consumption during and immediately after coronary artery bypass and valve surgery, and to correlate these changes with pharmacologic therapy and clinical events.



Project Description: Patients undergoing elective coronary artery bypass surgery will be studied. For coronary blood flow measurement, a coronary sinus thermodilution catheter will be advanced into the great cardiac vein (GCV) via the right internal jugular vein under fluoroscopic guidance one hour prior to the surgical procedure. In addition, as is routine practice, a Swan-Ganz catheter will be inserted into the pulmonary artery via left internal jugular vein and an arterial line will be placed in the radial artery. Once all catheters are in place, the following measurements will be recorded: arterial blood pressure, cardiac output, mean pulmonary capillary wedge pressure, and great cardiac vein blood flow and resistance. GCV blood will be sent for measurement of lactate, oxygen content and catecholamines. Baseline arterial and mixed venous blood gases, electrolytes, and systemic catecholamines and lactate will also be measured. The patient will be taken to the operating room, anesthetized, and prepped for surgery in the usual fashion. No detail in the management of the patient during surgery will be different from that routinely followed, except for the periodic measurements and blood samples taken as outlined below.

Upon induction of anesthesia, during the operation, and during the first postoperative hours, blood will be drawn from the great cardiac vein and systemic artery and analyzed for oxygen content, lactate content, and circulating catecholamine levels. Great cardiac vein blood flow and cardiac output will also be determined at the same intervals. Other blood samples, such as arterial blood gases, electrolyte levels, hemoglobin and hematocrit, clotting studies, etc., will be done for the usual clinical indications and routine monitoring purposes. The following are the times that GCV and systemic blood will be sampled and GCV blood flow and cardiac output determined:

1. immediately prior to induction of anesthesia
2. 15 minutes after of induction of anesthesia
3. just prior to instituting cardiopulmonary bypass
4. during sternal closure after the patient is removed for CPB
5. at 1 hour intervals in the ICU for 8 hours

The purpose of the first three measurements is to determine what changes in cardiac blood flow and metabolism occur by the induction of anesthesia and operation. The measurements in parts 4 and 5 will determine changes in coronary blood flow and cardiac metabolism after removal from cardiopulmonary bypass and postoperatively during the first six to twelve hours, at which time adverse events attributable to coronary artery spasm have occurred in patients recovering from coronary artery bypass grafting. At the end of the study interval, the coronary sinus catheter will be removed. The Swan-Ganz catheter will be left in place according to the needs of the patient.

Accurate and meaningful measurements of coronary blood flow depend on proper positioning of the catheter. In the operating room, a stable position of the catheter will be verified by palpation. A radiopaque marker will be sewn to the epicardium overlying the position of the proximal electrode in the GCV to make verification of position by fluoroscopy in the recovery room, a simple and rapid process. All patients in the study will be on a fluoroscopic bed. All measurements will be taken near end expiration during normal respiration or mechanical ventilation because of the effect of respiration on the accuracy of measurement.





Attempts will be made to control as many hemodynamic variables as possible in the postoperative period. Pulmonary capillary wedge pressure will be kept  $\pm 5$  mm Hg from preoperative values. The mean arterial pressure will be maintained at 70-80 mm Hg. Atrial pacing will be used to keep the heart rate at 90. All of these measures are routinely employed in the postoperative coronary artery bypass patient.

Proposed Course: Experience has been gained by the investigators in the use and proper positioning of these thermodilution catheters in animal models. The proposed human studies are now beginning.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02742-04 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Intracardiac Assessment and Utility of New Ultrasonic Technologies

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Scott T. McMillan, Ph.D., Post Doctoral Fellow, Georgia Institute of Technology  
Ajit P. Yoganathan, Ph.D., Assistant Professor, Georgia Institute of Technology  
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Cardiovascular Fluid Dynamics Laboratory, School of Chemical Engineering  
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## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The development of new modes of Doppler ultrasound technologies and new developments in the methods of signal electronic processing of the received ultrasonic spectra permit accurate assessments of velocities of intracardiac blood flow. Color-encoded, two-dimensional Doppler permits qualitative and quantitative evaluations of flow field velocity patterns and estimates of turbulence profiles. Studies performed utilizing these technologies include the following: 1) Doppler velocity/flow mapping in vivo and in vitro of clinical and preclinical prosthetic mitral valves; 2) comparison of in vivo Doppler ultrasound with in vitro Doppler ultrasound and laser Doppler anemometry; 3) in vivo determinations of velocity, flow turbulence profiles of prosthetic valves and comparisons made to in vitro observations as they relate to shear stresses, making correlations with clinical events; 4) assessments of mitral regurgitation; 5) evaluation of the efficacy of mitral annuloplasty techniques; and 6) observations upon normal left ventricular diastolic velocity/flow events to better understand normal native mitral valve hemodynamic physiology.



Project Description: The development of ultrasound systems with multiple frequencies integrated with continuous and pulsed wave Doppler, simultaneous color-encoded, two-dimensional and M-mode imaging in real time and with 10 ms ECG gating in one device, has opened a new field of non-invasive accurate quantitation of a variety of normal physiological and pathophysiological hemodynamic conditions. The clinical applications include non-invasive in vivo velocity, flow and turbulence visualization. These types of observations should add substantially to our understanding of the hemodynamic pathophysiology of normally functioning and malfunctioning prosthetic heart valves. The observations also have implications regarding improvements in prosthetic valve designs.

In the past, flow visualization techniques for the study of prosthetic heart valves have been limited to in vitro systems. However, the development of color-encoded, two-dimensional Doppler technology enables the visualization of velocity/flow and turbulence relationships of intracardiac events in vivo under physiological, non-invasive conditions.

During the past two years, more than 180 (120 during the current year) Doppler velocities/flow studies of 32 different types of clinical and preclinical valves have been performed after implantation in the mitral position in juvenile sheep. An additional ten studies have been performed after mitral valve ring annuloplasties. Sixty-eight studies were performed late, i.e. 20 weeks after implantation; the remainder were performed early (0 to 14 days). The valves studied included 10 types of porcine aortic bioprosthetic valves, 8 types of tilting disc mechanical valves, 3 types of ball and cage mechanical valves, 1 type of bileaflet hemidisc mechanical valve, and 6 types of preclinical synthetic leaflet valves.

Color Doppler velocity/flow and turbulence profiles were imaged in real time and with ECG gating (Aloka system SSD-880). The study protocol including imaging at 10 ms intervals throughout ventricular diastole and systole. Two transducer carrier interrogation frequencies (2.5 and 5 MHz) and 3 pulsed repetition frequencies (4, 6, and 8 kHz) were used for a total of 5 maximal resolvable velocities without aliasing from 31 to 121 cm/s (overall range of 4 to 121 cm/s). Velocities which aliased in the pulsed modes were quantitated utilizing continuous wave Doppler with an independent transducer (Pedof) or a dual function transducer. Three or four orthogonal planes were used. Studies were performed at 3 heart rates ranging from 60 to 120/min and at cardiac outputs ranging from 2 to 4.5 l/min, which represented resting, normally active and hyperactive hemodynamic states.

Color Doppler studies of 20 normal native mitral valves have added substantially to the understanding of velocity/flow events occurring during left ventricular diastole. At the onset of aortic valve closure, its closing volume was visualized occupying the left ventricular outflow tract for a distance of 1 to 2 cm below the aortic valve and having velocities in the range of 40 cm/s lasting no more than 10 to 20 ms. During isovolumic left ventricular relaxation, the mitral valve apparatus moved forward for a duration of approximately another 10 ms without the leaflets opening, but producing a low (10 to 30 m/s) velocity displacement front of blood towards the left ventricular apex. Within another 10 to 20 ms at the onset of passive filling, the valve leaflets began to open and the blood velocity accelerated and became mildly turbulent. A small region of vortex shedding with flow reversal



appeared behind the anterior mitral valve leaflet in the left ventricular outflow tract. By mid diastole, both leaflets were widely open and a broad, laminar, low velocity (not exceeding 60 cm/s) profile of blood extended from the left atrium to the left ventricular apex. Flow occupied 100% of the anatomic cross-sectional mitral orifice area. Later in diastole, blood displaced from the left ventricular apex appeared as regions of flow reversal along the left ventricular free wall and septum producing partial closure of the mitral valve leaflets. Following atrial systole, left ventricular inflow again accelerated with velocities usually exceeding 60 cm/sec and the mitral valve leaflets again opened, although not so far as during passive left ventricular filling. In late diastole, flow reversals reappeared produced by displacement of blood from the left ventricular body and apex, causing near closure of the mitral valve leaflets prior to the onset left ventricular systole. Because of this event, with the onset of left ventricular mechanical systole the mitral valve closed with a minimum closing volume, and with a duration of no more than 10 ms. These in vivo observations demonstrate the efficiency of physiological events during normal ventricular diastole and have implications regarding the dysfunction of prosthetic mitral valves and implications regarding future prosthetic valve designs.

In contrast to the above in vivo observations are those of various types of prosthetic mitral valves.

Porcine aortic bioprosthesis valves had high velocity (maximal 150 - 200 cm/s), turbulent, eccentric jets. Areas of flow separation and vortex formation appeared beneath the leaflets during ventricular systole. Bovine pericardial bioprosthesis valves had central, but often asymmetric, high velocity jets with in-orifice turbulence which decayed 1-2 cm downstream. Maximal velocities generally were lower than for the porcine aortic valves. However, for both porcine aortic and bovine pericardial bioprosthesis valves, flow areas (as determined by Doppler velocity profiles) occupied only 50-75% of the cross-sectional, orifice inflow areas. New generation (low pressure fixed and low profile) types of bioprosthesis valves now undergoing clinical trials demonstrated better velocity/flow characteristics than the old generation (currently used clinically) bioprosthesis valves.

Standard and color-encoded, two-dimensional imaging and Doppler studies were performed on 28 tilting disc valves of eight types implanted in the mitral position in sheep. Valves were oriented with the major orifices to the left ventricular free wall or to the left ventricular septum. Tilting disc valves in the mitral position have better (more nearly normal physiologically) velocity profiles with the major orifice oriented to the left ventricular free wall than to the septum.

Ball and cage and disc and cage mechanical valves had very turbulent, high velocity (150-200 cm/s), peripheral jets. Flow areas were asymmetric, being substantially less than 50% of the cross-sectional, orifice inflow areas, and less than 25% for some of the disc and cage valves. All cage valves had wakes producing areas of negative velocities distal to the occluders, which were larger in area and more severe (velocities in excess of 50 cm/s) for the disc and cage valves.

Bileaflet, hemidisc valves had jets from the lateral two orifices which appeared larger than that through the central orifice; the former jets measured





between 80 and 120 cm/s. Color spectrum variance, indicating turbulence, was present laterally.

Closing velocities, and intrinsic and perivalvular regurgitation were detectable for the native, bioprosthetic and synthetic leaflet valves, but thus far have proved difficult to image for the mechanical valves because of ultrasound attenuation and reverberations caused by the occluders during ventricular systole. However, the regurgitant velocities occurring with mechanical valves were localized and quantified with the continuous wave Doppler mode.

The studies performed in vivo in the animal model system were compared with velocity/flow and turbulence studies performed in vitro in a pulse duplicator system, in vitro flow visualization studies and in vitro laser Doppler anemometry studies.

The in vitro pulsatile flow studies were conducted in a model of the left ventricle with identical types of valves used for the in vivo animal studies. Under similar hemodynamic conditions to the in vivo studies, velocity and turbulence profiles were measured using standard pulsed and continuous wave Doppler, and color-encoded, two-dimensional Doppler. These studies were compared to those velocity and turbulence characteristics determined by two-dimensional laser Doppler anemometry. Both porcine aortic and bovine pericardial bioprostheses demonstrated jet type flow fields with maximal velocities in the range of 150 cm/s; regions of flow reversals in the range of -60 cm/s were seen lateral to the jets. Regions of high turbulence were confined to narrow areas at the edges of the jets. With ball and cage valves high velocity (greater than 100 cm/s) jets were seen circumferential to the occluder, the jets being greater between the ball and the left ventricular free wall than between the ball and the left ventricular outflow tract. Reverse velocities (-50 cm/s) occurred in the central part of the flow channel distal to the occluder as a result of flow separation and vortex eddy formation. Disc and cage valves demonstrated similar types of flow fields, although the peripheral jets were smaller in volume and the volume of the central reverse velocities were larger. Tilting disc valves showed velocities as high as 150 cm/s through the major and minor orifices with regions of stagnation, and flow separation and reversal, immediately distal to the disc. The bileaflet valves showed three jets, but most of the flow occurred through the two lateral orifices. An area of high turbulence was present in the region between the jet and the left ventricular free wall.

Thus, the types of velocity/flow patterns and turbulence profiles observed in vivo and by two types of in vitro Doppler techniques under physiological, hemodynamic conditions are comparable. Color-encoded, two dimensional Doppler techniques do permit in vivo evaluation of normally functioning prosthetic cardiac valves and should prove useful for detecting and understanding the clinical problems associated with these valves.

There was a linear relationship between the imaged area of the regurgitant jet and the regurgitant volume. However, using the 2.5 MHz interrogation frequency, the appearance of the jet differed and the slope of the interrelationship of calculated regurgitant volume versus imaged regurgitant area was significantly higher for the Toshiba system ( $r = 0.92$ ; slope =  $21 \text{ cm}^2/\text{cm}^3$ ) compared to the Aloka system ( $r = 0.88$ , slope =  $12.5$



cm<sup>2</sup>/cm<sup>3</sup>). Color flow imaging of mitral regurgitation appears to be dependent upon a number of instrumentation factors, including the type of system, as well as the gain settings, pulse repetition frequency and transducer carrier frequency within any one system.

Proposed Course: The collaborative studies with Dr. Yoganathan and Dr. McMillan to further compare in vivo and in vitro Doppler flow visualization techniques will continue. Other types of clinical and preclinical valves will be studied and correlations with clinical performance will be made. Studies of closing and regurgitant volumes associated with mechanical valves will be made using a transesophageal transducer and a left atrial window. The utility of these types of ultrasound technologies for evaluating the efficacy of mitral valve annuloplasty techniques will be explored. Further studies are planned to continue to investigate the velocity, flow and turbulence events occurring during the cardiac cycle under normal and abnormal physiologic states. New uses are being investigated to make possible clinical assessments of valvular and other cardiac abnormalities.

#### Presentations

Jones M, Eidbo EE, McMillan ST, Eidbo JP, Sandlin JL, Yoganathan AP, Clark RE: Tilting disc orientation for prosthetic mitral valves: in vivo Doppler studies. 59th Scientific Sessions. American Heart Association, November, 1986.

Hoit B, Jones M, Eidbo EE, Meyer T, Valdes-Cruz LM, Sahn DJ, Elias W: Evaluation of regurgitant jets in an animal model with quantifiable mitral insufficiency using color flow, mapping Doppler. 59th Scientific Sessions. American Heart Association. November, 1986.

Yoganathan AP, Jones M, Sahn DJ, Ridgeway A, Jimoh A, Tamura T: Bernoulli gradient calculations for mechanical prosthetic aortic valves: In vitro Doppler studies. 59th Scientific Sessions. American Heart Association. November, 1986.

Jones M, Hoit B, Eidbo EE, Meyer T, Sahn DJ, Elias W: Factors affecting color flow mapping Doppler imaging of regurgitant jets in an animal model of mitral insufficiency. 35th Annual Scientific Session. American College of Cardiology. March, 1987.

McMillan ST, Woo Y-R, Jones M, Eidbo EE, Yoganathan AP: In vitro/in vivo velocity and turbulence characterization of mitral heart valve prostheses. 1987 Biomechanics Symposium. June, 1987.

McMillan ST, Woo Y-R, Jones M, Eidbo EE, Yoganathan AP: In vitro and in vivo velocity and turbulence comparisons of mitral heart valve prostheses. Northeastern Bioengineering Conference. March, 1987.

#### Publications:

Yoganathan AP, Woo A-P, Sung H-W, Williams FP, Jones M: In vitro hemodynamic characteristics of tissue bioprotheses in the aortic position. J Thorac Cardiovasc Surg 92:198-209, 1986.

Jones M, McMillan ST, Eidbo EE, Woo Y-R, Yoganathan AP: Evaluation of prosthetic heart valves by Doppler flow imaging. Echocardiography.



2:513-525, 1986.

Sherman FS, Valdes-Cruz SM, Sahn DJ, Elias W, Jones M, Hagen-Ansert S, Scagnelli S, Swenson RE: Two-dimensional Doppler flow mapping for detecting atrial and ventricular septal defects: Studies in animal models and in the clinical setting. In: Pediatric Cardiology: Proceedings of the Second World Congress of Pediatric Cardiology. (Doyle EF, Engle MA, Gersony WM, Rashkind WF, Talner NS, Eds): 201-204, Springer Verlag, New York, 1986.

McMillan ST, Woo Y-R, Jones M, Eidbo EE, Hall L, Yoganathan AP: Velocity and turbulence measurements in the vicinity of mitral prosthetic valves using ultrasound Doppler. In: 1987 Biomechanics Symposium. (Blutler DL, Torsilli PA, Eds): 41-44, The American Society of Mechanical Engineers, New York, 1987.

Jones M, Eidbo EE: Experimental laboratory use of color Doppler: Emphasis on its use for the evaluation of prosthetic cardiac valves. In: Atlas on Color Doppler. (Nanda NC, Ed): Lea & Febiger, Philadelphia, (in press).

Yoganathan AP, Sung H-W, Woo Y-R, Jones M: In vitro velocity and turbulence measurements in the vicinity of three new mechanical aortic heart valve prostheses. J Thorac Cardiovasc Surg (in press).

Yoganathan AP, Jones M: Advances in prosthetic aortic heart valves: Fluid mechanics of aortic valve design. J Biomat App (in press).

McMillan ST, Woo Y-R, Jones M, Eidbo EE, Yoganathan AP: In vitro and in vivo velocity and turbulence comparisons of mitral heart valve prostheses. Proceedings of the 1987 Northeastern Bioengineering Conference. (in press).

#### Abstracts Published:

Hoit B, Jones M, Eidbo EE, Meyer T, Valdes-Cruz LM, Sahn DJ, Elias W: Evaluation of regurgitant jets in an animal model with quantifiable mitral insufficiency using color flow mapping Doppler. Circulation. 74:11-131, 1986.

Jones M, Eidbo EE, McMillan ST, Eidbo JP, Sandlin JL, Yoganathan AP, Clark RE: Tilting disc orientation for prosthetic mitral valves: in vitro Doppler studies. Circulation. 74:11-394, 1986.

Jones M, Eidbo EE, Clark RE: Color Doppler flow mapping of prosthetic heart valves. Proceedings of the 39th Annual Conference on Engineering in Medicine and Biology. 136, 1986.

McMillan ST, Yoganathan AP, Mumpower EL, Eidbo EE, Jones M: In vitro characterization of prosthetic valves. Proceedings of the 39th Annual Conference on Engineering in Medicine and Biology. 137, 1986.

Yoganathan AP, Jones M, Sahn DJ, Ridgeway A, Jimoh A, Tamura T: Bernoulli gradient calculations for mechanical prosthetic aortic valves: In vitro Doppler studies. Circulation. 74:11-391, 1986.

Jones M, Hoit B, Eidbo EE, Meyer T, Sahn DJ, Elias W: Factors affecting color flow mapping Doppler imaging of regurgitant jets in an animal model of mitral



insufficiency. J Am Coll Cardiology. 9:64A, 1987. June, 1987.

Manuscripts in Preparation:

Experimental evaluation of prosthetic valve flow. For: Echocardiography and Doppler in Cardiac Surgery. (Maurer G, Ed).

An animal model for the evaluation of prosthetic valve function: Comparisons with in vitro studies. For: In Vitro Studies of Cardiac Flow and Their Application to Clinical Doppler Echocardiography. (Sahn DJ, Yoganathan AP, Eds).

Evaluation of mitral insufficiency using two-dimensional color Doppler flow imaging technologies.

Understanding left ventricular diastolic hemodynamic events. Contributions made by color-encoded, two-dimensional Doppler and implications regarding the function of prosthetic mitral valves.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02767-02 SU

## PERIOD COVERED

October 1, 1986 through May 1, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Operation for Hypertrophic Subaortic Stenosis in the Elderly

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Matthew M. Cooper, M.D., Clinical Associate, Surgery Branch, NHLBI  
Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Surgery Branch, NHLBI  
Eben Tucker, M.D., Cardiology Consultant, Cardiology Branch, NHLBI  
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hypothesis tested in this study was that the septal myectomy operation for palliative treatment of hypertrophic cardiomyopathy was equally worthwhile in those 65 years and older as has been shown for younger patients. 52 patients for whom complete data were available for >5 years after the operation or until death comprised the study. The mean age was 69 ± 3 years (range 65-81) 57% of whom were female. 41 patients had no coronary artery disease (CAD) requiring surgical palliation. The remainder had CAD. 82 and 95% reduction in peak left ventricular outflow pressure gradients was achieved in the two groups, respectively. An 8% hospital mortality and 75 ± 8% 5 year survival was achieved in those without CAD. Those with CAD had increased hospital mortality and lower 5 year survival rates. These data support the clinical impression that older age is not a contraindication to operative palliation although the presence of significant CAD increases risk.



Project Description: A previous report of the older population ( $\geq 65$  year) was published more than a decade ago. As the average age of patients with HCM who have an operation has gradually increased over this interval, the issue of hospital and long-term mortality and presence of coronary artery disease was examined. The Surgical-Cardiology data base of more than 10,000 patients was culled to meet entry criteria. These were: age  $\geq 65$  year, complete pre and postoperative data on all hospital survivors and complete long-term follow up of all patients. Data were compiled from the records of hemodynamic, angiographic, ECG and ultrasonic studies, exercise testing, clinical examinations. The mean age was  $69 \pm 3$  year (range 65-81) and 39 were female. 11 patients had concomitant coronary artery bypass procedures. The mean follow up interval was 54 months (5-120). A reduction of the left ventricular outflow tract pressure gradient  $65 \pm 16$  to  $3 \pm 1$  mm Hg and  $95 \pm 13$  to  $17 \pm 9$  mm Hg was achieved in those with and without CAD, respectively. New conduction defects were found postoperatively in 65% of survivors, 85% of whom were improved one or more NYHA Classes at follow-up 6-12 months after operation. The mean preoperative class was  $3.2 \pm 0.1$  and at latest follow up (1986) was  $1.9 \pm 0.1$ . The hospital mortality rate was 8% for those without CAD which is identical to that for patients less than 65 years of age both historically and for those operated upon during the same time interval. Those that had CAD had a 27% hospital mortality rate. All 3 of the 11 with CAD-HCM patient deaths were associated with the operative creation of an interventricular septal defect which was poorly tolerated by the elderly especially those with CAD.

The data demonstrate that the elderly have the same degree of palliation and operative and 5 year survivorship as those that are younger. The creation of an IVSD in patients with CAD has resulted in a very careful approach to these patients including opting for mitral valve replacement if necessary.

Presentation: Presented at the American College of Cardiology Annual Meeting 1986.

Publications: Accepted for publication. Annals of Thoracic Surgery 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02768-02 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of the Interaction of Heparin with Heparin Binding Growth Factor-I

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Todd K. Rosengart, M.D., Clinical Associate, Surgery Branch, NHLBI

Robert Friesel, Technologist IV, Molecular Biology Lab, Holland Lab, American Red Cross, Rockville, MD

Tevie Mehlman, Technologist IV, Molecular Biology Lab, Holland Lab, American Red Cross, Rockville, MD

Wilson H. Burgess, Technologist IV, Molecular Biology Lab, Holland Lab, American Red Cross, Rockville, MD

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (If any)

Molecular Biology Lab, Holland Lab, American Red Cross, Rockville, MD

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

$I^{125}$ -ECGF recently redesignated heparin binding growth factor-I (HBGF-I) has a very short half-life. These studies have shown that heparin markedly inhibits proteolytic digestion of HBGF by trypsin and other proteases. This property was lost after thermal denaturation of HBGF, suggesting a heparin - HBGF structural interaction rather than a heparin - trypsin interaction is responsible for the trypsin resistance of HBGF. These data suggest that heparin ameliorates HBGF denaturation and provides conformational stability to the polypeptide growth factor. The stabilizing effect of heparin was dependent upon the concentration of heparin as well as temperature and duration. Autoradiography of  $I^{125}$ -HBGF incubated with human umbilical vein endothelial cells demonstrated near complete inhibition of proteolytic digestion of HBGF when the incubation was performed in the presence of heparin. Inhibitor experiments suggest this serum derived protease is of the metallo-protease class. These data support the concept that the mechanism of the heparin-induced human endothelial cell phenotype involves the protection of HBGF by heparin against inactivation by endothelial cell-derived proteolytic enzymes.

Subsequent studies have extended these observations to heparin sulfate, the predominant form of the glycosaminoglycan in the extracellular matrix of the vessel wall, and further suggests a biologic role for this interaction. Low molecular weight heparin fragments with markedly decreased anticoagulant effects also protect HBGF-I, which will be useful for the use of HBGF-I as an infusable agent. Heparin fragments also preserved the bioactivity of HBGF-I maintained at 37°C.

806



Project Description: HBGF isolated from bovine brain was iodinated as previously described and subjected to trypsin digestion for 3 and 24 hours. Autoradiography was performed according to standard practice, yielding a characteristic pattern of HBGF digestion bands. Pre-treatment of HBGF with commercially available heparin, heparan sulfate or low molecular weight heparin fragments and thermal denaturation of HBGF in a standard water bath yielded results as described above. Human umbilical vein endothelial cells were grown in standard media without HBGF or heparin. One or both agents were added and allowed to incubate overnight. The media was then extracted and subjected to protein isolation on C<sub>18</sub> Sep-Pak columns (Waters), SDS-PAGE analysis and autoradiography. Thymidine assay of cell replication in these cultures was performed according to standard practice.

Projected Course: Further analysis and classification of the serum derived proteases will be performed. A determination of whether heparin modifies HBGF-I intracellularly as well as extracellularly will be made.

Presentations: American Society of Biological Chemistry, Philadelphia, PA, June 1987.

Publications: Manuscript accepted for publication by Journal of Cell Biology 1987.





## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02769-02 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Heparin Fragments & Heparin Binding Growth Factor-I Interaction in Vivo

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Todd K. Rosengart, M.D., Clinical Associate, Surgery Branch, NHLBI

John Kupferschmid, M.D., Clinical Associate, Surgery Branch, NHLBI

Victor Ferrans, M.D., Senior Investigator, Pathology Branch, NHLBI

Gerald Kelly, Research Assistant, Surgery Branch, NHLBI

Ward Cassells, M.D., Medical Staff Fellow, Cardiology Branch, NHLBI

Ellis Unger, M.D., Medical Staff Fellow, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Pathology Branch, NHLBI

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.0

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A multiphased analysis in an animal model of the effects of non-anticoagulant heparin fragments and heparin binding growth factor-I (HBGF-I), was undertaken in three parts using a balloon injury model. The three parts of the analysis include: a) study of the pharmacokinetics and distribution of HBGF-I in a rat; b) determination of binding characteristics of HBGF-I to intact and injured rat vasculature, and c) examination of the ability of HBGF-I to accelerate endothelial regeneration and act synergistically with heparin. HBGF-I causes acceleration of endothelial cell growth in vitro which is enhanced by heparin.

The data demonstrate a rapid HBGF-I half-life which is prolonged by heparin. Heparin enhances HBGF-I binding to all organs measured except kidney, which is decreased. Saturation of binding occurs at an approximate blood concentration of 10 ng/ml. Heparin fragments with HBGF-I appear to increase endothelial regrowth into denuded rat carotid artery approximately 2-fold.



Project Description: Preliminary assessment of HBGF-I pharmacokinetics and bioactivity were made in rats by measuring blood and organ levels following intravenous bolus. Balloon injury to carotid artery was performed as previously described in order to denude endothelium. Binding studies were performed by counting injured and uninjured carotid arteries which were harvested following intravenous bolus of  $^{125}\text{I}$ -HBGF-I with or without heparin. Endothelial regrowth into denuded carotid vessels was assessed by scanning EM and Evan's blue staining following 7 day infusion of heparin fragments with HBGF-I or heparin fragments alone administered with an implantable infusion pump.

Presentations:

American Foundation for Clinical Research, San Diego, CA

May, 1987.

Society for Vascular Surgery, Toronto, Canada, June, 1987.

Publications:

Journal of Vascular Surgery, in press.

Journal of Clinical Investigation, in preparation.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02772-02 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Use of Fenoldopam in Postcardiotomy Low Output Syndrome

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Surgery Branch, NHLBI

David E. Webb, M.D., Expert Consultant, Office of the Director, CC

## COOPERATING UNITS (if any)

Nephrology Branch

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The effect of the selective dopamine receptor agonist fenoldopam on renal perfusion, renal function and cardiac function and cardiac hemodynamics will be assessed in 30 patients who demonstrate low cardiac output syndrome (LCOS). Ten patients without low cardiac output syndrome following open heart operation will serve as controls.

Fenoldopam is an investigational agent that is 6x as potent as dopamine in causing renal vasodilatation. It should be an ideal agent in reversing the renal hypoperfusion thought to play a significant role in acute renal failure following post-cardiotomy low output syndrome. Fenoldopam also causes systemic vasodilatation and should provide effective afterload reduction.



Project Description: Post-operative patients will be monitored by standard techniques including pulmonary artery catheter and will also be evaluated by inulin and PAH clearance determinations, to determine glomerular filtration rate and renal blood flow, respectively. Upon fulfilling specified baseline entry conditions, patients will be assigned to LCOS or non-LCOS treatment groups. Fenoldopam will be infused until a maximal rate of 1.5 mg/kg/min is reached, or until certain hemodynamic parameters are fulfilled. A two hour infusion and data collection period will ensue, followed by an infusion weaning period and followup data collection.

Proposed Course: Clinical trial should last approximately 12 months, from 8/87 to 8/88. Due to difficulties in initiation of project, one patient has received drug to date, with favorable initial results. Patient was subsequently dropped from protocol secondary to unstable heart rhythm probably unrelated to investigational drug use.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02774-02 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hemodynamic and Ultrasonic Studies of Prosthetic Heart Valves In Vitro

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Francisco Arabia, M.D., Clinical Associate, Surgery Branch, NHLBI

Thomas L. Talbot, M.M.E., Biomedical &amp; Instrumentation Branch

Michael Jones, M.D., Senior Investigator, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

NONE

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hypotheses tested in these in vitro studies were: a) continuous wave Doppler (CWD) provides accurate assessment of pressure differences across prosthetic valves as a function of flow and b) color-encoded, two-dimensional Doppler aids in the interpretation of traditional flow visualization. A physiologic pulse duplicator with pressure and electromagnetic transducers, an IBM AT microprocessor, and ALOKA-SSD-880 ultrasonic system were used. Twelve mechanical aortic prostheses (1 ball valve, 2 bileaflets, and 3 tilting disc, sizes 23 & 27 mm) were studied. Flow visualization studies used a 35 mm camera, laser light source with a planoconvex lens 90° to axial flow, and amberlite particles (420 M) in normal saline solution. Peak pressure differences were measured at 1 cm increments from 1 to 6 cm distal from the valve sewing ring. Simultaneous maximal velocities were obtained with CWD and transformed to pressure differences with the simplified Bernoulli equation. Manometric peak pressure differences decreased from the 1 cm (range  $P = 3.0 - 51.2$  mm Hg) to the 6 cm (range  $P = 1.2 - 33.0$  mm Hg) loci. Maximal velocities (range 4.88 m/s to 1.21 m/s) varied by valve but corresponded with maximal manometric data. Linear regression between the Doppler derived and measured pressure differences showed slopes for each valve ranging from 0.71 to 1.62, and an intercept ranging from -14.5 mm Hg to 10.5 mm Hg;  $0.95 < r < 0.99$ . Flow visualization and color flow imaging revealed similar flow patterns for valves with similar geometrical configurations. It is concluded that pressure differences across prosthetic valves are a function of the location of measurement and that CWD is an accurate method of determining maximal pressure differences at known flow and cycle rates. Color flow imaging provides new accurate information about flow patterns. Ultrasonic technology provides new dynamic information about the in vitro performance characteristics of prosthetic valves.



Project Description: Since the development and use of prosthetic heart valves, numerous in vivo and in vitro techniques have been used to compare the performances of these valves. In vitro circulatory systems have been used to determine flow patterns, regurgitation, and pressure differences. Currently, cardiac catheterization is the clinical means utilized to evaluate patients after valve replacement. Recently, Doppler echocardiography has become a useful, noninvasive, diagnostic method for the study of cardiac anatomy and physiology. Numerous investigators have demonstrated its accuracy by comparing the results obtained from patients with normal and diseased native heart valves to those obtained during cardiac catheterization. Others have demonstrated that the simplified Bernoulli equation:

$$P_2 - P_1 = 4V_2^2$$

where  $P_2 - P_1$  = pressure difference  
and  $V_2$  = velocity of a fluid past in area of narrowing

can be used to determine the pressure difference across a stenotic valve orifice. These experiments were the first in a series to evaluate the performance of prosthetic heart valves in vivo and in vitro with Doppler echocardiography. The hypothesis to be tested is that Doppler echocardiography is an accurate method to determine pressure differences across prosthetic heart valves in vitro.

An in vitro circulatory system (Dynatek, D-11) capable of accepting prosthetic heart valves in the aortic and mitral position is used. This system is capable of reproducing physiologic flow rates and pressures. Twelve currently clinically utilized prosthetic heart valves: Starr-Edwards 1260 (23 & 27 mm), Bjork-Shiley (23 & 27 mm), St. Jude (23 & 27 mm), Medtronic-Hall (23 & 27 mm), Omniscience (23 & 27 mm) and Duromedics (23 & 27 mm) were studied. Testing conditions were: stroke volume  $70 \pm 1$  cc, temp =  $21 \pm 2^\circ\text{C}$  and mean aortic pressure 95-100 mm Hg. The heart rate was set at 60, 80, 100, and 120 bpm. Sheep blood was used as the testing fluid; the hematocrit was maintained at  $11 \pm 2\%$ . An Aloka 880 ultrasonic Doppler system with color flow imaging capability is used to determine the velocity of the blood as it passes across the prosthetic valve. The system can also be used for flow pattern visualization. This utilizes normal saline rather than blood and a laser light source.

Results: Pressure differences across the prosthetic heart valves were manometrically measured at 1 cm, increments from the valve seat and parallel to flow. It was clearly demonstrated that pressure differences decreased as the measurement point moved away from the valve. It was also demonstrated that the maximum pressure difference occurred within the most proximal 2 cm of the valve.

The velocity of the blood during pulsatile flow was measured in the continuous wave mode. The velocities were then transferred to a pressure term as described. The correlation coefficient (r) between the Doppler derived pressure difference and the maximal manometric pressure difference for each of the twelve valves was  $0.95 < r < 0.99$ . Linear regression showed that the slopes were between 0.8 and 1.6, the slopes varied for all valves. The Aloka 880 ultrasonic system is capable of generating visualization of flow patterns by assigning a color to different velocities. These flow patterns were then



compared to those obtained with the laser light and were extremely similar. It has been shown that Doppler ultrasound can be used to determine pressure differences across mechanical aortic prosthetic heart valves at known flow rates in vitro. Prior knowledge of the valve orientation is essential in order to determine the maximum pressure difference. Ultrasonic color flow imaging is an accurate method determining the flow pattern generated by the valve under pulsatile conditions in vitro.

Proposed Course. The next step of this investigation will involve the study of bioprosthetic valves under similar physiologic conditions as described. This will be followed by the study of prosthetic heart valves in the mitral position with and without atrial contractions.

Presentations: Abstract submitted to the Southern Thoracic Surgical Society.

Publications: Three manuscripts have been prepared and are under review.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02776-01 SU

## PERIOD COVERED

October 1, 1986 through June 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Effect of Left Ventricular Septal Myectomy on Concurrent Mitral Regurgitation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Matthew M. Cooper, M.D., Clinical Associate, Surgery Branch, NHLBI

Eben Tucker, M.D., Cardiology Consultant, Cardiology Branch, NHLBI

Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

1

OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The hypothesis tested was that left ventricular septal myectomy improved coexisting mitral regurgitation. The pre- and postoperative cine left ventriculograms of 39 patients were compared. The mean difference in time between studies was 15 months. Approximately one half of the patients had a reduction in mitral regurgitation. There was no association between relief of obstructive pressure gradient at rest or with provocation or symptoms and improvement in mitral regurgitation.

815





Project Description: One of the benefits of the palliative operation devised by Morrow for the treatment of hypertrophic cardiomyopathy in highly symptomatic patients was thought to be a reduction in the amount of mitral regurgitation present, to some degree, in 75% of the surgical population. The observation was logical in as much as most patients had pressure gradients greater than 50 mm Hg at rest or with provocation. Hence, reduction in the peak left ventricular systolic pressure should reduce the volume of blood regurgitant through the mitral valve during the systolic interval of the cardiac cycle. However, not all patients restudied by cardiac catheterization techniques had a change in the amount of mitral regurgitation as semiquantified by cine left ventriculography despite complete relief of the outflow obstruction. To determine the incidence and the degree to which left ventricular myectomy/myotomy improved preexisting mitral regurgitation, a study of 29 patients was undertaken.

The Surgery - Cardiology Branch data base of more than 10,000 patients was used. Repeat left ventriculography has been a recent addition to the postoperative study of patients with hypertrophic cardiomyopathy. Data were sought for years 1981-1987 so that all patients would have had the procedure by the same surgeon, (CLM). Additional entry criteria were presence of mitral regurgitation preoperatively and high quality resolution of both cine angiograms for comparison. The cines were randomized (pre vs. post) and all were independently reviewed by two observers (MMC and ET). Scores for mitral regurgitation (1-4+) were matched and, where different, a third person provided consultation. Scores of 2 or more grade differences between observations on the same patient were considered significant. Mitral regurgitation due to catheter entrapment, arrhythmia or intrinsic mitral valve disease were exclusion criteria. Hemodynamic status and symptomatic status were also evaluated.

The data demonstrated that 2 of 8 (25%) patients with mitral regurgitation, coronary artery disease, and HCM had improvement in the degree of regurgitation. In contrast, 16 of 31 patients (52%) without coronary artery disease had significant improvement in mitral regurgitation ( $p < 0.01$ ). This result suggests that the mechanism of mitral regurgitation in HCM is compounded by papillary muscle dysfunction caused by ischemic heart disease which is not altered by coronary artery bypass grafting. Comparison of hemodynamic data for those without ischemic heart disease failed to show significant differences in the reduction of the peak gradient differences in NYHA functional class pre- or postoperatively. All groups were significantly improved by at least two NYHA functional classes and all had adequate procedures with  $\geq 90\%$  reduction of left ventricular outflow tract obstruction as determined by pressure-flow measurements at both catheterizations. These results suggest that excess systolic pressure within the left ventricle of patients with HCM is only a contributing factor to mitral regurgitation, not the major mechanism. Previous geometric anatomic studies of the relation of the mitral valvular apparatus diastolic and systolic axes of the left ventricle have demonstrated marked differences compared to normal hearts. Hence, this study in conjunction with past ones suggest that mitral regurgitation is a consequence of this genetic disease's manifestation in abnormal geometry and not solely the result of left ventricular hypertension caused by outflow tract obstruction.

Proposed Course: A manuscript presently in draft form is under review and



revision by the authors. All patients in this study are to be followed by the Surgery Branch in the clinic . When clinically indicated or in five years, all will be asked to participate in a third catheterization and a determination will be made with respect to the natural history of mitral regurgitation in this patient population. All those dying before restudy will have careful study of their hearts by the Pathology Branch, NHLBI.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02777-01 SU

## PERIOD COVERED

July 1, 1986 through

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Use of Monoclonal Anti-Tac Antibody as Immunosuppression for Cardiac Xenografts

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Matthew M. Cooper, M.D., Clinical Associate, Surgery Branch, NHLBI

Thomas A. Waldman, M.D., Chief, Immunology Branch, NCI

Otto Gansow, Ph.D., Head, Inorganic and Radioimmunochemistry Section,  
Radiation Oncology Branch, NCI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

Immunology Branch, NCI

Inorganic and Radioimmunochemistry Section, Radiation Oncology Branch, NCI

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study is designed to evaluate the effectiveness of three forms of anti-Tac, a monoclonal antibody that recognizes the interleukin-2 (IL-2) receptor as sole immunosuppressive therapy in a model of heterotopic primate cardiac xenografting. The IL-2 receptor is not expressed on resting cells but is expressed on T-cells activated by interaction with foreign transplantation antigens. Unconjugated anti-Tac and anti-Tac chelated to Pseudomonas exotoxin and to <sup>90</sup>Yttrium (Y-90), a beta emitting isotope, are evaluated for efficacy and safety. Analysis of graft survival, modification of the composition of specific T-cell phenotypes, and toxicity were performed by comparing animals receiving no immunosuppression (N=3) with those receiving the various forms of anti-Tac (N=15). Five animals receiving anti-Tac only failed to have increased survival. Five of 7 Rhesus receiving the exotoxin died of toxicity. One of three animals receiving Y-90 was alive and well two weeks after implant with no other immunosuppression. The evaluation of possible enhanced efficacy of the monoclonal chelates over the unmodified form as well as toxicity accrued will have direct relevance to human use of such agents in organ transplantation and in the treatment of adult T-cell leukemia, a leukemia expressing the Tac antigen.



Project Description: The animal system of cardiac transplantation used a heart from a cynomolgus monkey (*M. fascicularis*) implanted in a Rhesus (*M. mulatta*) recipient with which it is cross-match negative. Animals are sedated and anesthetized with inhalation agents and the electrocardiogram and arterial pressure continuously measured. The donor heart is harvested following arrest with cold oxygenated crystalloid cardioplegia and application of ice slush. An atrial septal defect is created and the mitral valve is rendered incompetent to prevent ventricular distention. The right and left atrial orifices are closed and the heart is sutured to the right common carotid artery and internal jugular vein of the recipient. The heart fits well subcutaneously and is readily visible and palpable.

Three control animals received no immunosuppression and rejected their grafts at  $7.3 \pm .3$  days. Fluorescent activated cell sorter analysis (FACS) failed to reveal a large proliferation of Tac positive cells at the time of rejection. However, soluble IL-2 receptor levels increased approximately nine-fold over baseline at the time of rejection. Electrocardiographic and echocardiography were found not to be more sensitive than inspection and palpation in the detection of rejection. Rejection in all animals was confirmed by myocardial biopsy. The recipient monkey is able to eat and move later on the day of operation.

Unmodified anti-Tac was given in multiple doses to five animals. Graft survival averaged  $6.8 \pm 1.2$  days and was not prolonged compared to control animals. Soluble IL-2 receptor cannot at present be measured in the presence of anti-Tac. Modification of the assay to allow this is currently underway. Mouse IgG levels in the serum (a measure of anti-Tac) were detected at markedly increased levels following administration of anti-Tac. Antibody generated against anti-Tac itself was not detectable in the serum until the ninth postoperative day.

A Pseudomonal exotoxin conjugate to anti-Tac was given to seven animals. Rejection occurred in two animals on days four and seven. Five animals expired due to toxicity related to the exotoxin. This occurred despite previous human and primate trials with a similar exotoxin which demonstrated acceptable levels of toxicity.

Three animals to date have received  $^{90}\text{Y}$  chelated to anti-Tac. One animal rejected his graft at five days after receiving 1.2 mCi. A second animal rejected his graft at seven days after receiving a total of 4.96 mCi divided between two doses. A third animal received a total of approximately 16 mCi divided among four doses. It's graft remains functional without evidence of rejection 16 days following transplantation. The only toxicity demonstrated has been a selective depletion of the lymphocyte pool in the animal receiving 16 mCi of  $^{90}\text{Y}$ -anti-Tac. This may merely represent adequate function of the isotope chelate at this dosage or represent the effect of beta emission only.

Unmodified anti-Tac is insufficient to prolong xenograft survival though it has proven effective in a primate renal allograft model. A Pseudomonal exotoxin conjugate of anti-Tac may or may not prolong graft survival. However, toxicity precludes further human or animal use at this time until further modification of the toxin is accomplished.  $^{90}\text{Y}$  - anti-Tac if given in





adequate quantity shows promise in prolonging xenograft survival. Soluble IL-2 receptor shows promise as a noninvasive indicator of rejection, the validity of which must be further substantiated.

Proposed Course: An additional five animals are to receive  $^{90}\text{Y}$ -anti-Tac in doses of 4 cmCi each. If prolongation of graft survival is substantiated then a similar set of animals will receive  $^{90}\text{Y}$  bound to a nonspecific antibody such as UPC-10 or RPC - 5 to verify that the effect is specific and due to the anti-Tac rather than a generalized effect of the beta-emission. Further toxicity and localization studies employing escalating doses of  $^{90}\text{Y}$ ,  $^{88}\text{Y}$  and  $^{111}\text{In}$  will be carried out in animals that have previously rejected their grafts. Serial determinations of Mouse IgG and antibody to anti-Tac will be followed in all animals even after graft rejection to determine the kinetics of both of these. Modification of the assay for soluble IL-2 receptor to allow detection of in the presence of anti-Tac is currently under way.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02778-01 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of Colloid Versus Crystalloid Fluid Administration on Lung Water during CPBP

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Marc E. Mitchell, M.D., Clinical Associate, Surgery Branch, NHLBI

Julie A. Swain, M.D., Senior Surgeon and Senior Investigator, Surgery Branch, NHLBI

Matthew M. Cooper, M.D., Clinical Associate, Surgery Branch, NHLBI

Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

NONE

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Forty adult patients undergoing elective surgical procedures requiring cardiopulmonary bypass will be studied in the perioperative period including the 24 hours immediately following operation. The hypotheses to be tested are: 1) measures of extravascular lung water and colloid osmotic pressure can be used to predict the postoperative clinical respiratory status of patients; and 2) the use of a colloid rather than a crystalloid solution for perioperative fluid resuscitation affects postoperative respiratory function and the need for respiratory support, hemodynamic variables, and cost. Possible adverse effects associated with either fluid resuscitation regimen will be determined.

f21



Project Description: Controversy persists as to the optimal type of fluid resuscitation during and after cardiopulmonary bypass (CPB). CPB has been shown to cause loss of integrity of the pulmonary capillary membrane. Fluid replacement with albumin has been recommended to preserve intravascular volume and thereby limit extravasation and interstitial fluid accumulation and, in contrast, has been thought deleterious by as increasing interstitial accumulation by crossing the compromised pulmonary capillary membrane. Crystalloid has been advocated due to the lower cost as compared with albumin and the failure to find clinical correlates of the decrease in colloid osmotic pressure (COP) associated with its administration. It is also argued that there is a the need to measure COP in order to prevent increases in extravascular lung water (EVLW) which compromise pulmonary function. Incomplete and conflicting data from a variety of different model systems and limited data in the context of clinical CPB have prompted this investigation. The type of fluid resuscitation employed has direct clinical bearing on postoperative patients, particularly those undergoing procedures that require CPB in which some breach of pulmonary capillary integrity is known to occur.

The hypotheses to be tested are : 1) the EVLW and COP can be used to predict the postoperative clinical respiratory status of patients; and 2) the use of a colloid versus crystalloid solution for perioperative fluid resuscitation affects postoperative pulmonary function. These hypotheses will be tested by evaluating respiratory function and the need for respiratory support, hemodynamic variables, cost differentials, and adverse effects associated with either fluid resuscitation regimen.

Extravascular lung water is quantitated using a thermal-green dye double indicator dilution technique employing an automated American Edwards Lung Water Computer. This technique involves simultaneous injection of two indicators into the right atrium. One indicator remains entirely intravascular while the other diffuses freely across the pulmonary capillaries to mix with the extravascular water in the lungs. The difference between the distribution volumes of the two indicators represents the extravascular lung water volume. Colloid osmotic pressure is measured directly using a Wescor Colloid Osmometer whose operation is based upon the transudation of water molecules and diffusible solute particles from a reference chamber to the sample chamber across a synthetic, semipermeable membrane until hydrostatic equilibrium is achieved.

Patient Selection and Data Collection: Twenty patients with and twenty without secondary pulmonary hypertension will be divided equally among the following four groups for study and analysis: 1) patients with secondary pulmonary hypertension receiving colloid fluid resuscitation; 2) patients with pulmonary hypertension receiving crystalloid resuscitation; 3) patients without pulmonary hypertension receiving colloid resuscitation; and 4) patients without pulmonary hypertension receiving crystalloid resuscitation. Patients with secondary pulmonary hypertension are defined as those with pulmonary artery systolic pressure  $> 50$  mm Hg and/or increased pulmonary vascular resistance defined as  $> 5$  Wood's units at the time of preoperative catheterization. The absence of secondary pulmonary hypertension is defined as pulmonary artery systolic pressure  $\leq 30$  mm Hg with a normal pulmonary vascular resistance ( $\leq 3$  Wood's units).



Hemodynamic data obtained at the preoperative catheterization will be used to divide patients into those with and without pulmonary hypertension only if this catheterization is performed within one month of operation. For patients whose preoperative catheterization is performed earlier than one month prior to operation, hemodynamic data obtained at the time of anesthetic induction will be used to classify patients with regard to pulmonary hypertension.

Randomization of patients to receive either colloid or crystalloid will be done by selection of a sealed envelope from a pool which will initially contain an equal number of colloid and crystalloid envelopes totalling the number of patients required for completion of this protocol.

Data collection includes chest radiograph (PA and LAT), hemodynamic data obtained at cardiac catheterization, and hemoglobin level for use in calculations.

Prior to anesthetic induction, the pulmonary hypertensive and normal lung patients are randomized with respect to receiving colloid or crystalloid fluid resuscitation. During the period of induction, routine placement of a Swan-Ganz pulmonary artery catheter and peripheral venous and arterial access are performed.

Following routine sterile prep and draping, a Femoral Artery Lung Water Catheter is placed percutaneously in the femoral artery. Baseline values of EVLW, COP, serum and urine osmolality, cardiac output, mean pulmonary artery pressure (PA), pulmonary artery systolic (PAS), diastolic (PAD), and mean wedge (PAW) pressures, and  $paO_2$  are measured and cardiac index, pulmonary and systemic vascular resistances, COP-PAW gradient, intrapulmonary shunt fraction ( $Q_s/Q_t$ ), and A-a gradient are calculated. The tidal volume will be maintained at 12 cc/Kg to allow estimation of the relative pulmonary compliance. Repeat measurements and calculations are performed: 1) just prior to instituting CPB prior to systemic heparinization, and 2) during placement of the sternal wires while closing the median sternotomy. All measures of EVLW are performed in duplicate or until reproducible within 10% of each other.

Repeat measurements of EVLW are performed in the ICU one hour after returning from the operating room and then every 6 hours for 48 hours.

All data will be analyzed to determine if significant differences in any of the above measured or calculated variables result from colloid versus crystalloid fluid resuscitation. Any differences in the duration of intubation and respiratory support will be determined as well as differences in the incidence of subsequent pneumonia. The total volume of resuscitation and postoperative weight gain will be tallied for each patient. The total amount of fluid given will be corrected for body surface area to avoid the influence of size on fluid requirements. Any cost differential between colloid and crystalloid fluid resuscitation will be determined as well as adverse effects attributable to either method.

Data: To date four patients have completed the study: one patient with pulmonary hypertension who received colloid, one patient with pulmonary hypertension who received crystalloid, and two patients with normal pulmonary pressures who received crystalloid fluid resuscitation. Several other patients who were enrolled in the study had to be excluded for a variety of reasons.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02779-01 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

NMR Studies of Hypothermia, and Intracellular pH of the Heart and Brain

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Julie A. Swain, M.D., Senior Surgeon &amp; Senior Investigator, Surgery Branch, NHLBI

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## COOPERATING UNITS (if any)

Laboratory of Kidney and Electrolyte Metabolism

## LAB/BRANCH

Surgery Branch, NHLBI

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The effect of acid-base status on intracellular pH and high energy phosphate content during hypothermia is unknown in warm-blooded animals. What is known is that changes in intracellular pH have widespread implications for metabolic functions of the cell. The variation of intracellular pH with temperature has been well characterized by comparative physiologists for hibernators and ectothermic animals. However, no data has been accumulated on the changes in intracellular pH of the heart and brain in warm blooded animals. This study has important implications for the conduct of cardiac surgery and hypothermic cardiopulmonary bypass in man.

The hypothesis to be tested is that by maintaining pH parallel to the neutral pH of water (that is, increasing pH during hypothermia), the intracellular pH of the heart and brain will also follow the neutral pH of water and that this will be beneficial for the cellular energy state. An additional hypothesis is that a correlation can be found between two methods of pH measurement (NMR and the NIH pH probe).

The experimental model consists of 8 to 10 week old sheep. This model was chosen because sheep past this age have very little 2,3-DPG in the blood. The Nuclear Magnetic Resonance (NMR frequency of inorganic phosphate reflects intracellular pH. Because 2,3-DPG resonates at a frequency near inorganic phosphate, it is necessary to have a model free of 2,3-DPG with a radiofrequency coil on either the heart or brain. The animal will be cooled using surface cooling in the bore of the magnet. Nuclear magnetic resonance spectra will be used to assess the changes in intracellular pH and in phosphorylation potential during hypothermia as a function of blood pH for the heart and brain.



Project Description: The two most common pH management schemes encountered during hypothermia are termed pH-stat and alpha-stat. The term pH-stat is used when pH is kept constant at all temperatures and is the scheme that describes the behavior of arterial blood in hibernating animals during hypothermia. In contrast, ectotherms (cold-blooded) animals vary their temperature with the environment. Their blood pH is related to body temperature by the formula:

$$\text{pH/Temperature} = -0.015/^{\circ}\text{C}$$

This relation also describes the behavior of blood in a sealed tube at constant total carbon dioxide, which is analogous to the conditions encountered by blood after leaving the pulmonary capillaries when perfusing the body tissues which are at various temperatures. In addition, this pH-temperature relation describes the behavior of the neutral pH of water.

In ectothermic animals, blood and tissue pH follow the alpha-stat scheme. Although the blood of hibernators follows the pH-stat scheme, the heart and liver (essential organs for preservation of the organism during hibernation) follow the alpha-stat scheme. An active proton pump mechanism, which remains to be characterized, must maintain a hydrogen ion gradient between blood and tissues. This relatively acidotic state of blood during hypothermia represents an adaptation to respiratory acidosis by the hibernating mammal. No evidence exists whether man or other heterothermic animals such as sheep have any of the specialized adaptative mechanisms that are present in hibernators. In fact, no measures of intracellular pH during hypothermia have been made in heterothermic animals. The present investigation will determine if sheep are able to maintain this pH gradient during hypothermia in the brain or heart.

Measurement of Intracellular pH: Various methods have been utilized for the measurement of intracellular pH. In past studies by comparative physiologists in hibernators, intracellular pH was determined by using the distribution of the weak acid radioactive tracer DMO (dimethyl-oxazolidine, dione). The disadvantages of this classical method are that it requires sacrifice of the animals and can only be used for one measurement.

Nuclear magnetic resonance spectroscopy (NMR) can be used to measure intracellular pH in vivo. The frequency of the  $\text{p}^{31}\text{-NMR}$  signal of inorganic phosphate is sensitive to pH in the physiologic region. Around neutral pH, inorganic phosphate exists as two species ( $\text{HPO}_2^-$  and  $\text{H}_2\text{PO}_4^-$ ) with different NMR signal frequencies. These two species exchange with each other rapidly, resulting in the frequency of the single NMR signal being determined by the relative amounts of the two species and being proportional to the intracellular pH. This method is highly accurate because of the relatively large amount of intracellular inorganic phosphate in the brain. However, in the well-oxygenated myocardium, the amount of inorganic phosphate is relatively low and pH measurements require equipment capable of high resolution. For this reason these studies utilize a wide-bore CSI 4.7 Tesla magnet to provide acceptable resolution.

The third method of measuring intracellular pH is by implantation of direct electrodes. Because of cardiac motion, standard microelectrodes cannot be used. A miniature transducer for simultaneous measurement of pH and temperature has been designed at NIH. The needle pH sensor (0.6 mm) penetrates the myocardium or brain and measures tissue pH (a combination of



intra- and extracellular pH). The method of measurement is based on the change in light absorbance of an indicator dye (phenol red) with changing pH. This probe has been used by the Cardiac Surgery Branch to follow ischemia and has correlated well with other measures of ischemia. With the current availability of a wide-bore, high field strength NMR, the correlation between tissue pH (as measured by the NIH probe) and intracellular pH (by NMR) will be made. This correlation is necessary to aid in the interpretation of data generated by use of this probe to assess myocardial ischemia and intracellular pH in future experiments planned in humans. NMR can be used to measure the concentrations of ATP, PCr and Pi in tissues. An estimation of the energy potential of the tissue can be obtained by the ratio  $[PCr]/[ATP]$ .

Experimental Protocol: Twenty sheep over the age of 8 weeks will be used in these studies. The animals are anesthetized with Pentothol and maintained with Halothane, nitrous oxide, and Pavulon. A circulating water heating blanket will be used to maintain rectal temperature at 38°C during the surgical preparation and initial measurements.

A left thoracotomy is performed for placement of the radiofrequency coil on the left ventricle and the pH probe on the left ventricle. The scalp will be removed to facilitate placement of the RF coil on the cranium. A femoral artery catheter and a peripheral intravenous catheter are inserted.

After instrumentation, the animal is placed in the bore of the magnet and intracellular pH and phosphorylation potential by NMR and pH by probe is determined.

The cooling blanket is used to surface cool the animal slowly to 25°C. The ventilatory minute volume is adjusted to provide the selected pH scheme during hypothermia. Intravenous xylocaine (2 mg/kg) is given prior to cooling to prevent ventricular fibrillation. The heart is paced to maintain heart rate at 60 bpm. After the temperature of the animal has been stabilized at 25°C for 15 minutes, the above measurements are repeated and the experiment is terminated.

Data: Multiple NMR experiments on normothermic dogs were performed at different levels of blood pH. Because the NMR resonance of inorganic phosphate (Pi) is overlapped by that of 2,3-DPG in the blood, intracellular pH of the heart in the dog could not be precisely determined, even with high resolution NMR instrumentation. This was confirmed by replacing the animal's blood with oxygen carrying Flurosol, which demonstrated the overlap of Pi and 2,3-DPG. Several preliminary experiments on sheep have found the above described protocol to be suitable for these experiments.

Proposed Course: The CSI 4.7T, 40 cm bore magnet will be used for future experiments on adolescent sheep. It is expected that these experiments will be completed within 6 months and that a total of 20 animals will be needed to complete the experiments.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 02780-01 SU

## PERIOD COVERED

October 1, 1986 through September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Effect of Hypothermia on Myocardial Tissue pH during Cardiopulmonary Bypass

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Robert C. Robbins, M.D., Clinical Associate, Surgery Branch, NHLBI

Julie A. Swain, M.D., Senior Surgeon, Surgery Branch, NHLBI

Thomas McDonald, B.S., Technician, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Surgery Branch

## SECTION

## INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The effect of hypothermia on intracellular myocardial pH in humans is unknown. The acid-base status of blood during systemic hypothermia theoretically should have profound effects on myocardial tissue pH. Variations in myocardial tissue pH affect enzyme function and the ability of the organ to withstand ischemia. The purpose of this project is to determine whether myocardial pH varies with changes in blood pH.

The NIH tissue pH probe has been inserted in the myocardium of 16 patients during hypothermic cardiopulmonary bypass. The acid-base scheme followed was either that of alpha-stat (N=3) or pH-stat (N=11) and the tissue pH was compared to that of the myocardium using these two pH management schemes. Two patients had inadequate data. The preliminary results show that the changes in myocardial pH parallel those of the blood pH. Additionally, the pH-stat scheme appears to result in a small elevation of pH such that the myocardium is acidotic during hypothermia. The study is to continue.

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Project Description: The majority of cardiac surgical procedures involve the use of systemic hypothermia in an attempt to decrease the metabolic activity of various organ systems. The ideal arterial pH during hypothermia has not been firmly established. One method used to regulate arterial pH, pH-STAT, maintains the pH at 7.40 for all temperatures.

The Rosenthal equation establishes the relationship between pH and temperature as: the change in pH per degree centigrade is equal to  $-0.015$ . This relationship applies to the behavior of the neutral pH of water as well as to the changes in pH of blood in a sealed system at constant carbon dioxide levels. According to this equation, an arterial pH of 7.40 at  $20^{\circ}\text{C}$  would represent a significant acidosis when measured at  $37^{\circ}\text{C}$  ( $\text{pH}=7.145$ ). The second method, alpha-stat, follows the pattern of cold-blooded animals, ectotherms, by varying arterial pH with temperature in accordance with the Rosenthal equation. With this scheme of pH regulation, a pH of 7.40 is optimal only at thirty-seven degrees centigrade and pH increases as temperature decreases resulting in a pH relationship that is parallel to the neutral pH of water during hypothermia.

The pH STAT scheme is achieved during cardiopulmonary bypass by the addition of carbon dioxide to the gas mixture to produce a respiratory acidosis and thus the maintenance of an arterial pH of 7.40 with decreasing temperature. Conversely, the alpha stat scheme allows for decreasing levels of carbon dioxide from the gas mixture with decreases in temperature to produce a respiratory alkalosis and the maintenance of arterial pH values that correspond to changes set forth by the Rosenthal equation. In humans the changes in myocardial pH during hypothermia have not been clearly established.

In ectothermic animals, blood and tissue pH follow the alpha-stat scheme. Although the blood of hibernators follows the pH-stat scheme, the heart follows the alpha-stat scheme. An active proton pump mechanism, which remains to be characterized, must maintain a hydrogen ion gradient between blood and tissues. This relatively acidotic state of blood during hypothermia represents an adaptation to respiratory acidosis by the hibernating mammal. No evidence exists whether man or other heterothermic animals have any of the specialized adaptive mechanisms that are present in hibernators. In fact, no measure of intracellular pH during hypothermia has been made in heterothermic animals. The present investigation will determine if man is able to maintain this pH gradient during hypothermia.

The purpose of this study is to investigate changes in myocardial pH in man during hypothermic cardiopulmonary bypass employing either the pH stat or alpha stat method of arterial pH regulation.

Measurement of Myocardial pH: Simultaneous and continuous measurement of interstitial myocardial pH and temperature is accomplished with utilization of the NIH pH probe. The probe consists of a miniature circular transducer with two needle sensors. Each sensor has an outside diameter of 0.6 mm and a length of 5 mm. The pH sensor is based on the concept of measuring the color of a pH sensitive dye contained within a hydrogen ion permeable envelope placed in the tissue to be analyzed. The probe consists of two single-strand filaments of polystyrene inserted in the bore of a 23 gauge needle that is sealed at the tip. A side orifice near the distal end of the needle is filled with indicator dye (phenol red) covalently bound to polyacryl-amide



microspheres which provides an open hydrophilic gel structure for ionic diffusion. Light emitted from a LED passes down one filament with the reflected light from the sensor passing back to a photodetector system via the second filament. Signals from the photo-multiplier tube are sent to a computer which provides digital pH values for each of a maximum of five pH probes. Tissue pH values are proportional to the ratio of green to red light absorbed by the sensor. Accuracy of the system is  $\pm 0.01$  pH units. Three standard phosphate buffers between pH 6.5 and 7.6 are used for calibration. The probes are calibrated immediately before and after each study. Values within 0.05 pH units of each reference buffer are used as the acceptable range of accuracy. A microprocessor algorithm based on predetermined constants relating isothermal pH change to temperature over the range of 0 - 40°C is used for the continuous correction for interstitial temperature changes. The probes are placed in a solution of Cydex for 16 hours prior to use to achieve sterilization.

Protocol: Patients undergoing elective cardiac surgery are included in the study. After anesthesia is induced, the chest is opened via a median sternotomy with arterial and venous cannulas placed and connected to the heart-lung machine. During this process the sterile pH probes are calibrated in the three reference buffers. The probes are inserted into the anterior and posterior right ventricular myocardium and shielded from room light. The pH readings are allowed to stabilize over 5 - 10 minutes. Cardiopulmonary bypass and systemic hypothermia are then initiated with continuous pH and temperature readings being recorded with a digital printer connected to the computer via a serial interface. The arterial blood perfusate pH and temperature are recorded at the beginning of cardiopulmonary bypass and with each 2°C decrease in myocardial temperature until ventricular fibrillation occurs. The pH transducer is then removed and the operation proceeds in the usual manner. The probes are then recalibrated with reference buffers.

Results: A total of sixteen patients have been studied with data from two patients not utilized due to technical difficulties with the pH sensor. The pH regulation of eleven patients was by the pH-stat method and three with the alpha-stat scheme. There were nine males and four females with eight patients undergoing coronary artery bypass grafting and six patients receiving valve replacements.

The pH increased an average of 0.124 units for the alpha-stat group compared to 0.069 units for the pH-stat group over similar changes in myocardial temperature from 25°C to 35°C. Approximately twenty more patients need to be studied to achieve statistically meaningful data.

It appears that during hypothermic cardiopulmonary bypass, if the arterial pH is maintained at 7.40 according to the pH-stat scheme, then the myocardium is acidotic relative to the neutral pH of water. Thus, this preliminary data demonstrates that the changes in myocardial pH parallel these changes in blood pH and that humans cannot maintain a blood-tissue hydrogen ion gradient.

Further studies will be needed to determine the significance of myocardial pH on myocardial function and metabolism following rewarming and reperfusion.



ANNUAL REPORT OF THE  
LABORATORY OF TECHNICAL DEVELOPMENT  
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

OCTOBER 1, 1986 TO SEPTEMBER 30, 1987

Separation Science Instrumentation

Development of the countercurrent chromatography (CCC) technology has been actively continued in various directions. Major efforts were directed toward innovation of instruments to extend both preparative and analytical capabilities of CCC. A novel scheme for preparative CCC called the cross-axis synchronous flow-through coil planet centrifuge produces a unique pattern of the centrifugal force field which enables reliable retention of the stationary phase with excellent phase mixing in a large-bore multilayer coil. High performance of the apparatus was demonstrated in gram-quantity separations of dinitrophenyl amino acids and dipeptides. A compact analytical high-speed CCC centrifuge was constructed to challenge feasibility of HSCCC/MS. The apparatus spins a narrow-bore (0.85 mm i.d.) multilayer coil at 2,000 rpm around 5 cm revolution radius. Our preliminary runs have demonstrated that the method is capable of separating microgram quantities of indole plant hormones at high partition efficiencies ranging from 1,000 to 1,300 theoretical plates within 90 minutes.

Efforts were also made to improve foam CCC by applying a long multilayer coil (40 m) equipped with five flow channels for dual countercurrent elution. The new column mounted on the cross-axis synchronous flow through coil planet centrifuge has produced chromatographic separation of two basic dyes according to a subtle difference in foam affinity.

Basic hydrodynamic studies were also continued with a newly designed rotary coil assembly which can accommodate a set of glass coils with different helical diameter (2.5 cm-20 cm). Hydrodynamic distribution of various two-phase solvent pairs observed in untreated and siliconized coils at various rotational speed indicated that the phase distribution is governed by interplay between two physical factors: solvent-wall interaction and Archimedean screw force.

Biophysical Instrumentation

The development of a new all-tantalum stopped flow microcalorimeter has proceeded to the point that a complete set of test data have been run and the instrument is now operational. Experiments are in progress on a number of DNA drug binding reactions. This work is being done in collaboration with scientists at Rutgers University. Only 80 microliters of each reagent are needed per reaction. The sensitivity is ten times that of the batch calorimeter requiring 1 ml of each reagent. For reactions with half-lives faster than 0.1 second, a reaction can run every 3 minutes. The instrument is controlled by a microcomputer which takes the data and analyzes it on



line. Extensive work with hemoglobin is planned for the coming year in collaboration with scientists at National Institute on Ageing, Letterman Army Research Institute, and Johns Hopkins University.

The adaptation of the isoionic hemoglobin preparation developed in this laboratory, by the joint Forces Casualty Care Blood Resources Division based at Letterman Army Research Institute as their reference standard has provided stimulus to a renewed interest in several instruments developed in this laboratory. A modified oxygen binding to hemoglobin apparatus has been set up and is presently undergoing tests in the Laboratory of Molecular Biology, NIA. A highly modified cell is nearing completion which will permit the measurement of the oxygen dissociation curve of hemoglobin simultaneously by a manometric method and spectroscopy. All of this work is directed toward the development of a stroma-free hemoglobin for emergency transfusions. Such data are needed if the various models of how hemoglobin binds oxygen are to be critically tested. In collaboration with computational scientists at UCLA and DCRT a considerable effort has been mounted to have all of the needed programs operating on the local IBM AT rather than a main frame computer.

The inertial drive quench flow apparatus and thermal-optical stopped flow apparatus have had a number of improvements made to them over the last year and it is expected that the quench flow machine will go operational in the laboratory of Biological Chemistry, NIA by the end of the summer. It has become clear that for the thermal stopped flow to be useful a differential version will need to be built and this should be finished in the next year. Experiments are planned on the ATPase ATP reactions of muscle as a test of the system.

#### Pulmonary and Cardiac Assist Devices

Through the application of new devices, new technologies, and the evaluation of experimental protocols in the animal research laboratory, the Section on Pulmonary and Cardiac Assist Devices seeked to enhance the management of acute pulmonary failure and acute cardiac failure. It was shown that mechanical pulmonary ventilation, as now clinically applied in the management of acute respiratory failure (ARF), can under some circumstances become highly injurious; such injury may become the overriding element that in time can lead to irreversible lung failure, multiorgan system failure, and death.

Healthy sheep, when exposed to mechanical pulmonary ventilation at a peak inspiratory pressure (PIP) of 50 cm H<sub>2</sub>O, will within 24 hours develop acute respiratory insufficiency, and go on to die. The evolution of the disease process is readily assessed from serial measurements in total static lung compliance (TSLC), functional residual capacity (FRC), and from arterial blood gases. Such lung failure, if allowed to progress to a TSLC of less than 25% of baseline values, is invariably lethal irrespective of treatment, either conventional continuous positive pressure ventilation (CPPC) or with extracorporeal membrane lung (ML) perfusion (16/16 died). Animals developed





multiorgan system failure, involving the renal, hepatic, CNS, the clotting system, in addition to total lung failure. Those studies convincingly show that mechanical ventilation (MV) at high PIP can lead by itself to a picture indistinguishable from clinical ARF.

When lung injury produced by MV is allowed to progress to where TSLC is reduced to 50% of baseline values, the resultant ARF still carries a high morbidity and only 3/11 sheep eventually recovered on conventional CPPV. However, when animals were placed on continuous positive airway pressure (CPAP) and all MV was discontinued (while metabolically produced CO<sub>2</sub> was removed by an extracorporeal ML perfusion system), all animals showed rapid improvement in lung function. Ultimately 9/11 sheep were weaned to room air, and on CPAP.

Our results point to an unrecognized danger from CPPV, particularly at near PIP used in this study. Our results suggest that those patients will likely do better if CPPV is discontinued, and the patient is placed on CPAP and extracorporeal ML to provide for CO<sub>2</sub> removal.

Previously, this Section has conducted studies on total cardiopulmonary bypass (CPBP) using a venous-arterial bypass system, incorporating a membrane artificial lung. Attempts at long term perfusion all failed.

In our present model, the heart is placed into ventricular fibrillation, while total CPBP is provided for by an extracorporeal ML perfusion system. During bypass, we now ventilate the lungs with 5% CO<sub>2</sub> in room air (and thereby avoid focal pulmonary capillary alkalosis); we affix a small spring "piggy-back" onto a Swan Ganz catheter, and place the spring at the level of the PA valve. Such placement assures that blood from the bronchial veins etc., can readily cross the lungs and drain into the right heart, without causing acute elevation in LA pressure. All blood is returned to the aorta in a pulsatile manner. Blood flow is very high, and exceeds 120 ml/kg min. Using such experimental methods, we were able to sustain total CPBP for 2-3 days with no pulmonary abnormalities, and recovery of total cardiac function in all animals so studied. Much longer bypass may well be feasible.

This animal model of total CPBP is effected through peripheral cannulation alone. This is an important consideration, when the alternative is implantation of an artificial heart as a bridge to transplant, or a left ventricular assist devices (LVAD) - both require thoracotomy both for insertion, and later removal of the device.

Our proposed system of total CPBP is applicable to right, left, or bilateral cardiac assist; or to pulmonary assist. Hence it covers the total realm of cardiopulmonary assist through peripheral cannulation alone.

#### Cell Culture and Calcium Measurement Systems

The porous bottom culture dishes (PBCDs) developed in this laboratory for the study of epithelial cells grown in confluent layers are now being used



in more than 500 laboratories world wide. This large utilization has resulted in part from the fact that PBCDs are now commercially available from 2 sources (Millipore Corp. and Costar Corp.) with several types of porous membranes and cell culture treatments and coatings. Many different types of epithelial and some endothelial and smooth muscle cells are being studied using the PBCDs. The objectives of these research projects which are being undertaken with the PBCDs go far beyond anything we had in mind at the outset of our development.

The PBCD made with collagen membranes also made in this laboratory, still provide the best optical properties (for observation of the cell layers with phase microscopy) of all the "homemade" and commercial PBCDs. However, the commercial PBCDs are appearing with increasingly good optical properties. The collagen membrane PBCDs have been very useful for growing endothelial and smooth muscle cells even though these cells produce very small potential differences and electrical resistances. In fact endothelial cells have been grown on one side of the collagen membrane and smooth muscle cells on the other side. This makes a good model of a blood vessel.

The devices developed in this laboratory to sterily study sodium transport by confluent layers of epithelial cells grown on the PBCDs are being used in many laboratories. As work with endothelial and other nonepithelial cells has increased, interest in these devices has developed for the measurement of electrical resistance of the cell layers as an indication of confluency or permeability to ions.

The fact that many cells in vivo grow on basement membranes which have permeabilities that are significantly smaller than those of the membranes used on the PBCDs in the past, has caused us to begin an investigation of reconstituted cellulose membranes which are often used for dialysis. Experiments spread over several years have shown that cell attachment to such membranes requires some sort of surface treatment. One treatment that gives cell attachment for many weeks at least is the brief exposure of the surface of the dialysis membrane to a methane-air flame. The optical properties of the resulting PBCDs are even better than the PBCDs made with "homemade" collagen membranes.

A major role played by Ca in the regulation of many cellular processes has prompted us to improve methods for measuring free Ca activity in cell systems. We have developed a 2 mm diameter electrode using a hydrophobic porous membrane and neutral carrier Ca exchanger which as a response time of 10 seconds and a resistance of 10 megohms. It is useful for measuring small quantities of solutions representative of cell interiors for many biochemical studies. These electrodes have also been useful in measuring interesting  $\text{Ca}^{++}$  activity reductions in perfusion solutions which apparently result from ion pairing of calcium and bicarbonate ions.

#### Laser Fluorescence Instrumentation

In time-resolved fluorescence spectroscopy, we have developed a new



instrument with unique capabilities. Nanosecond decay curves (useful to characterize the local conformation near tryptophans and tyrosines in proteins) often required hours to collect with previous instrumentation. Our instrument provides precise decay data now in a few seconds, opening up the possibility to study protein folding/unfolding and other fast reactions. Gating circuits are being added so we can proceed to even faster, "stopped flow" events. Meanwhile, the system has been exploited to examine stages in the unfolding of alcohol dehydrogenase, SH-controlled unfolding/refolding of thioredoxin and the chemical modification/dimerization of enzyme I. We have developed a multichannel detection scheme so multiple wavelengths and/or polarizations can be collected simultaneously, and new hybrid fluorometers (combined pulse/phase methods, two laser heterodyning, microwave alignment spectrofluorometry) are being prototyped at the same time.

The time resolved fluorescence of long-lived lipid probes (such as coronene) has recently provided us a direct look at bilayer packing fluctuations - - the transient, domain-melting effect that has been implicated in many membrane functions (anesthesia/channel gating, receptor mobility, messenger transduction, ion diffusion).

A large number of collaborative protein and lipid structural studies have also been undertaken; most take advantage of our laser-based spectrofluorometry and/or the continued developments in our "global analysis" programs.

### Fluorescence Spectroscopy

The work in fluorescence spectroscopy has been directed in two directions: 1. Problems in biophysics of intrinsic interest, and 2. Methods and techniques. One subject which was extensively studied was the mechanism of concentration quenching of fluorescent dyes encapsulated in liposomes. One such dye system, containing 6-carboxyfluorescein, is widely used to study liposome uptake and interactions. The dye is 97% quenched when inside the liposomes at a concentration of 0.2 M, but regains fluorescence when diluted. We obtained absorption spectra, fluorescence quenching data, fluorescence decay curves, and polarization data on the dye both free and encapsulated. Our conclusion was that the concentration quenching is due to formation of a nonfluorescent dimer and energy transfer to dimers. In contrast to some literature statements, no evidence of collisional quenching of the monomers was found.

The fluorescence of neutral 4-methylumbelliferone has been postulated to arise from a zwitterionic form which exists only in the excited state. Using our picosecond tunable laser system we found direct evidence for the formation of the zwitterion after excitation. This is an example of phototautomerism, which is ideally studied with our laser system. It should be possible to characterize the lifetimes, rates of formation, and fluorescence spectra of all the different molecular species, some of which may exist for only a few nanoseconds.



In the methods area, we completed our evaluation of a set of fluorescence standards lent to us by the National Bureau of Standards. These standards consisted of sintered Teflon disks containing inorganic phosphors whose emission spectra covered the visible region. The advantages of the standards included their stability to light and heat, and the fact that one could record their spectra and compare them to spectra obtained on the NBS reference spectrometer. The disadvantages were that the brass holders were slightly too large and had to be sanded down, there was interference from scattered and stray light which had to be corrected for, and variations in positioning of the standard samples and the Teflon blank. These findings were incorporated into a written report sent to NBS and may be made part of a joint communication.

The method for measuring association constants of metal ions and tryptophan peptides has been published. The method used for quenching of tryptophanyl fluorescence as the parameter to follow binding by  $\text{Cu}^{++}$  and  $\text{Ni}^{++}$ .

### Clinical Devices

The development of angioplasty catheters is continuing in cooperation with the Cardiology Branch. We have developed and tested in vitro an arc heated and a catalytic oxidation heated catheter tip that offer simplicity and economy over the use of the laser heated tip that has been clinically efficacious. Both of these can be made small enough for intracoronary use and are being commercially manufactured for clinical testing. Other applications of heat to angioplasty technology is being explored by resistive electrical heating and chemical heating by exothermic reactions.

A novel external counterpulsation device that stores energy in stretched rubber, exerts a rapid compression of the thighs, and is actuated by the house vacuum line to produce a compact economical method of obtaining cardiac support.

834





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01404-19 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Membrane Lungs for Long Term Respiratory, Cardiac and Cardiorespiratory Assist

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.	T. Kolobow	Medical Officer	LTD:NHLBI
	M. Borelli	Visiting Fellow	LTD:NHLBI
	F. Rossi	Visiting Fellow	LTD:NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

Section on Pulmonary and Cardiac Assist Devices

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

2

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Using animal models, we have shown that mechanical pulmonary ventilation as now practiced can under certain conditions become highly injurious, and lethal (adult respiratory distress syndrome = ARDS). This injury process is related to peak inspiratory pressures, and duration of mechanical pulmonary ventilation.

Using such an animal model of ARDS we have managed sheep on extracorporeal veno-venous blood gas exchange, while discontinuing all mechanical pulmonary ventilation. Within 24 hours of change in treatment to extracorporeal blood gas exchange, there was rapid improvement in pulmonary function in all animals, and 9/11 sheep were weaned off bypass and to room air. In a control group of animals treated with conventional continuous positive pressure ventilation (CPP) only 3/11 sheep could be weaned off bypass.

This animal model of ARDS can become sufficiently severe so that no form of presently available therapy will lead to survivors. Such understanding only emphasizes the as yet unrecognized dangers implied in the use of CPPV.

836



Project DescriptionObjectives:1. The Membrane Artificial Lung.

This laboratory was one of the earliest pioneers in the development of membrane artificial lung systems for short term, and long term applications. It is estimated, that world wide well over one half of all clinical cardiopulmonary bypass procedures are now carried out with systems that use membrane type artificial lungs.

The admittedly superior biologic and economic advantages of the membrane lung (ML) for short term use is greatly magnified by its use for long term laboratory and clinical applications. It is now possible to perform extracorporeal perfusions with a ML for pulmonary and cardiac assist lasting for days to one month. This capability has opened the possibility of providing such assistance through peripheral cannulation, hence requiring no thoracotomy.

2. To devise a safe extracorporeal perfusion system for extracorporeal removal of carbon dioxide in the control of alveolar ventilation, and in the control of breathing.

We have previously demonstrated that when a fraction of total metabolic CO<sub>2</sub> load is continuously removed by an extracorporeal ML system, alveolar ventilation will decrease in tandem. Such extracorporeal perfusion system can readily be used to avoid placing a patient on a mechanical ventilator (MV), or it can be used to immediately wean the patient off MV.

3. High pressure ventilation with a MV as a cause of acute lung injury.

It is not possible to selectively ventilate diseased areas of the lungs, the bulk of gas flow will invariably be directed to the most compliant parts of the lungs. Unfortunately, to attain adequate alveolar ventilation (so as to maintain PaCO<sub>2</sub> within normal range) often times requires high peak inspiratory pressures (PIP). We have postulated, that effects of high PIP may themselves cause deleterious pulmonary parenchymal and functional changes.



This study attempted to define the limits of MV at high PIP, and to show that recovery from lethal ARDS was possible when all MV was discontinued, and further management was with an extracorporeal ML perfusion system.

4. Demand cardiac assistance using an extracorporeal ML perfusion systems.

Total cardiopulmonary bypass (TCPBP) is routinely used in cardiac surgery, usually lasting a few hours. Beyond 9 hours, such procedures are associated with a prohibitive morbidity, and mortality. We hypothesized that safe TCPBP could be sustained for days, or weeks, provided pulmonary and cardiac management during TCPBP was handled in a special manner. Specifically, while pulmonary blood flow was markedly reduced, that the lungs continue to be ventilated with an equivalent of normal alveolar gases (room air with 5% CO<sub>2</sub>), and that right and left ventricular preloads be sustained within normal range at all times.

Methods employed and major findings:

1. The management of experimentally induced ARDS using an extracorporeal ML system.

a. In a model of ARDS induced by high PIP of 50 cm H<sub>2</sub>O, with total static lung compliance (TSLC) reduced by 75% or more.

All 11 16 sheep managed by either conventional treatment with continuous positive pressure ventilation (CPPV), or with extracorporeal ML bypass, died of multiorgan system failure, involving the pulmonary, renal, hepatic, CNS, and cardiovascular systems.

b. Of the 11 sheep with a milder form of ARDS induced similar to as under 1a, with TSLC reduced by 50%, all animals except 3 died of progressive multiorgan system failure while on CPPV; while all 11 sheep treated with extracorporeal ML perfusion showed marked improvement in lung function within 24 hours, and 9/11 could be weaned to room air.

Those results suggest great benefit derived from discontinuing high PIP MV. and instead places emphasis on extracorporeal ML bypass for partial, and total CO<sub>2</sub> removal. Such treatment, however, will not succeed, unless the adverse effects of MV are first acknowledged.

c. Preliminary studies strongly suggest that primary cardiac failure is more pervasive than previously thought in severest forms of ARDS. It appears likely that combining respiratory assist, with near total or total cardiac assist could overcome what appears to be a reversible cardiovascular collapse, and a diverse multiorgan system failure. If confirmed, such



treatment will greatly impact on current management of severest forms of ARDS.

2. We have succeeded in providing total CPBP in anesthetized, paralyzed sheep, with induced ventricular fibrillation, for 2-3 days, with full recovery following defibrillation. This success was keyed to three major technical items:

a. The development of a special spring-like device attached to a Swan Ganz catheter, which when placed in the pulmonary artery position, rendered the pulmonic valve incompetent. During cardiac arrest, such patent PA valve provided a ready means for bronchial and thebesian flow to drain from left to right, thereby decompressing the left heart, and maintaining normal PA pressures.

b. The native lungs were continuously ventilated with 5% CO<sub>2</sub> in room air, preventing pulmonary parenchymal alkalosis.

c. Special venous drainage catheters allowed high extracorporeal blood flows at from 100-150 ml/kg/min.

The ease of sustaining 3 days of total CPBP with full recovery (through peripheral cannulation alone) suggests that much longer periods of total CPBP may be achievable. Total body perfusion was sustained by the extracorporeal perfusion system. Organ function during bypass was excellent. There was no impairment in pulmonary function (one critical element to long term success), and cardiac recovery was complete. Such technique greatly reduces myocardial work, and is likely to find widespread clinical use.





Publications:

1. Kolobow, T., Solca, M., Chen, V., Buckhold, D.K., Pierce, J. E.: Enhancement of lung conditioning by acetylcholine in the prevention of respiratory distress syndrome in the preterm fetal lamb. Biol. Neonate 51: 224-233, 1987.
2. Kolobow, T.: Acute respiratory failure: on how to injure healthy lungs (and prevent sick lungs from recovering). ASAIO, in press, 1987.
3. Kolobow, T., Borelli, M., Spatola, R. Tsuno, K., and Prato, P.: Single catheter venous-venous membrane lung bypass in the treatment of experimental ARDS. ASAIO, in press, 1987.
4. Kolobow, T., Gattinoni, L., Pesenti, A., et al.: ECMO revisited. Int. J. Artif. Organs 10: No. 1, 1-2, 1987.
5. Kolobow, T., Moretti, M. P., Fumagalli, R., Mascheroni, D., Prato, P., Chen, V., and Joris, M.: Severe impairment in lung function induced by high peak airway pressure during mechanical ventilation. An experimental study. Am Rev Resp Dis 135: 312-315, 1987.
6. Foster, A. H., Kolobow, T.: A potential hazard of ventilation during early separation from total cardiopulmonary bypass. J. Thoracic and Cardiovasc. Surgery (letter to the editor) 93: 150-151, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01407-24 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Luminescence Spectroscopy in Biomedical Research

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.	R. F. Chen	Senior Investigator	LTD:NHLBI
	J. R. Knutson	Sr. Staff Fellow	LTD:NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Technical Development

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## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Some fluorescent dyes are largely quenched when incorporated into liposomes at high concentrations, but regain their fluorescence when released and diluted in solution. These dye-liposome systems have wide use in membrane and cell studies, but a plausible mechanism of the concentration quenching has not been given. The concentration quenching of various dyes encapsulated in phosphatidylcholine liposomes was studied. For 6-carboxyfluorescein, which is the most commonly used dye, no evidence for collisional quenching could be found. However, all the quenching observed at high dye concentration could be accounted for by nonfluorescent dimer formation and energy transfer to the dimer. Energy transfer could be demonstrated by polarization measurements, and dimerization could be quantitated by absorption spectra of the liposomes. A fraction of the dye was associated with the lipid membrane and not easily quenched either by energy transfer or by KI, a collisional quencher.

Studies on the fluorescence decay kinetics of 4-methylumbelliferone have provided direct evidence for the postulated occurrence of phototautomerism where a neutral ground state molecule becomes zwitterionic in the first excited state. Complex decay curves indicate the presence of transient intermediates whose spectra will be studied by the technique of decay associated spectroscopy, using our decay time fluorometer based on a tunable dye laser synchronously pumped by the doubled output of a Nd:YAG laser.

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Project DescriptionObjectives:

The purpose of the work is to solve specific problems of importance in biomedical research, relying mainly on various fluorescence spectroscopic methods. The specific problems are interesting, and the methods used will demonstrate novel spectroscopic techniques.

Methods Employed:

Spectra of absorption and fluorescence were obtained on Beckman and Cary spectrophotometers and Aminco-Bowman spectrofluorometers. Fluorescence decay kinetics were obtained on a single photon, time-correlated nanosecond fluorometer constructed with Dr. J. Knutson. The excitation source of the instrument is a synchronously pumped and doubled dye laser, pumped with a Nd:YAG laser. Purity of chemicals was assessed on an HP HPLC, while data reduction was achieved with a dedicated HP 900 minicomputer or the DCRT DEC-10 system, using MLAB.

1. The mechanism of concentration quenching of dyes in liposomes has been elucidated. The use of liposomes containing fluorescent dyes has become widespread ever since the demonstration of Weinstein et al (Science, 195, 489, 1977) that 6-carboxyfluorescein (6CF), encapsulated at high concentration, was quenched, but regained fluorescence when released and diluted. The dye-liposome system has been useful in measuring liposome fusion with cells or other liposomes, to follow uptake of liposomes by cells, to follow the enzymatic degradation of phosphatidylcholine in liposome walls, to measure phospholipid phase transitions, and a number of other applications. Despite the general use of this system, the mechanism of the concentration quenching is not generally known. We approached this question with spectroscopic and lifetime methods. It was found that, although the concentration quenching of xanthene-type dyes such as fluorescein, eosin, and rhodamine B was well known, there was no agreement in the literature as to the cause. Absorption spectra of dyes encapsulated in liposomes were found to be different from those of free dyes, indicating dimerization or higher aggregation. Spectral changes were noted not only for 6CF but also for Acid Fuchsin, Sulforhodamine B, other carboxyfluoresceins, and pyrene tri- and tetra-sulfonic acids. In the case of xanthene-type dyes, dimerization resulted in a nonfluorescent state, but could not account of the nearly total quenching of fluorescence. Even if the dimerization constant were as high as  $25 \text{ M}^{-1}$  only 60% of the dye would be dimerized at 0.2 M, whereas the fluorescence was 98% quenched. In addition, if dimerization were the sole cause of concentration quenching, the fluorescence lifetime would not be changed. In fact, measurement of fluorescence decay kinetics at 6CF



concentrations from 1 micromolar to .01 M indicated a progressive shortening of the 4.3 nsec lifetime. Fluorescence depolarization was noted as 6CF concentration in 90% glycerol was increased. Under these conditions, no significant rotational motion occurs during the fluorescence lifetime, and depolarization must be due to energy transfer. Calculations of the energy transfer distance, and the intermolecular distances at the concentrations used for liposomes, indicated that a reasonable mechanism of quenching was energy transfer to nonfluorescent dimers. Other workers have suggested that there additionally exists a collisional mechanism where monomers of dye collide and cause quenching. Experimentally, this phenomenon is difficult to approach because of the high optical density of concentrated fluorescein solutions, and because of self-absorption artifacts. Correction factors to compensate for these effects are high. We were able to measure fluorescence from .001 M 6CF by applying a correction for inner filter effects, and find no quenching in contrast to published reports. In addition, a fluorescein-like dye, phenolphthalein, was found to cause no quenching when added to 6CF. We concluded that a collisional quenching mechanism was unlikely, and quenching could be entirely accounted for by dimerization and energy transfer to dimers. In liposomes, 6CF also had a long lifetime component in its fluorescence decay. This was little changed by the presence of KI, a quencher. It was concluded that some 6CF associates with the lipid bilayer, thus accounting for the fact that fluorescence is not totally quenched in liposome-dye systems. The fluorescence of the entrapped pyrene sulfonates differs from that of the xanthene dyes, in that high concentration produces an excimer emission rather than simple quenching. Upon release from the liposomes, the excimer fluorescence disappears. The fluorescence decays of the pyrene sulfonates were measured. By understanding how concentration affects dye fluorescence, we can utilize dye-liposome systems more rationally and perhaps design other such systems.

2. The fluorescence decay kinetics of coumarin derivatives has been under study. These compounds include 4-methylumbelliferone, a common fluorophor used to create fluorogenic reagents. The interest in these compounds lies in the fact that they can exist in tautomeric forms, one of which is favored in the ground state, and another in the excited state. Thus, S. G. Schulman has postulated that 4-methylumbelliferone exhibits phototautomerism in which the ground state neutral form converts to a zwitterionic form upon excitation with light. This transformation would require the ejection of a proton from the 7-hydroxyl group and protonation of the carbonyl oxygen at position 2. It occurred to us that these events could be temporally resolved using the fluorescence lifetime apparatus. When 4-methylumbelliferone at neutral pH is excited at 300 nm with the dye laser, the fluorescence decay curve is unlike anything we have previously observed: there are at least 3 distinct kinetic events having lifetimes of .4, .8, and 4.8 nsec, and the .8 nsec component has a negative preexponential. Using decay associated spectroscopy, we hope





to obtain the spectra of these components. At present, it appears that the 4.8 nsec component represents the zwitterionic form, and the .4 nsec could be the neutral form. The species with the negative preexponential could be the molecule which has lost the proton from the hydroxyl group but not yet regained it at the carbonyl oxygen. These and other excited state reactions would seem to be ideally suited for study by our lifetime apparatus, which has picosecond resolution. Other compounds such as hydroxyquinolines and aminopyridines are thought to have excited state rearrangements of their protons, and could be studied by time-resolved fluorescence. Preliminary experiments show that the various lifetime components are wavelength dependent, so that the spectra of transient intermediates will be accessible by decay associated spectroscopy, a method which was largely developed by our colleague, Dr. J. Knutson.

#### Proposed Course:

The work on concentration quenching of dyes in liposomes excited considerable interest when presented at the Biophysics Society meeting in Feb., 1987 and is in the final stages of preparation for publication. Data on the dimerization constant for 6CF are being analyzed. We are also readying for publication data previously obtained on serotonin excited-state protonation and on the fluorescence properties tryptophan dipeptides. The newer observations of excited state proton rearrangements in umbelliferone derivatives will be vigorously pursued, as these phenomena have long been postulated but never directly observed, because of lack of suitable technology.

#### Publications:

1. R. F. Chen and J. R. Knutson, "Fluorescent Dyes Encapsulated in Liposomes: Mechanisms of the Fluorescence Changes", Biophysical J. 51: 539a, 1987 (abstract).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01408-22 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Methods in Fluorescence Spectroscopy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. R. F. Chen Senior Investigator LTD:NHLBI

## COOPERATING UNITS (if any)

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NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Fluorescent materials being proposed as fluorescence standards by the National Bureau of Standards have been evaluated regarding 1. stability, 2. intensity, 3. spectra, 4. interference from scatter, and 5. lifetimes. The lifetimes of these inorganic phosphors imbedded in sintered polytetrafluoroethylene were in the microsecond-millisecond range, so they would not be useful as lifetime standards for solution organic chemistry. However, the spectra, intensity, stability, and correctable scatter suggest that the materials could be used to calibrate and standardize fluorometers. Only mechanical defects (sample size, warping of the sheets) were significant.

The method for determining the association constants of tryptophan and tyrosine-containing peptides with cupric and nickel ions was developed and published. Quenching was the parameter used to quantitate binding. Interference from collisional quenching was considered to be negligible for studies of cupric ion binding, but could be a factor with nickel ion.

FYS



## Project Description

### Objectives:

The purpose of this project is to continue developing aspects of the technique of fluorescence spectroscopy, a useful method in biomedical research. The project includes assessments of standards, as well as the development of methods.

### Methods Employed:

Fluorescence spectra were recorded with Aminco-Bowman spectrofluorometers, and absorption was determined with a Zeiss spectrophotometer. Lifetimes were measured with a decay time apparatus based on a tunable dye laser synchronously pumped with a Nd:YAG laser. Chemicals were purchased commercially, while a set of fluorescence standards was lent to us by Drs. Weidner and Mavrodineanu of the National Bureau of Standards.

### Major Findings:

1. Evaluation of solid fluorescence samples from the National Bureau of Standards. Dr. Radu Mavrodineanu from the chemistry Division at N.B.S. asked us to collaborate in evaluating the properties of materials which may be proposed as fluorescence standards. Such standards are needed to check on instrumental fluctuations, and to calibrate the detector response of spectrofluorometers. Since the spectra of these materials has been recorded on the N.B.S. reference spectrofluorometer, other instruments could be calibrated using these materials. Other laboratories have cooperated in this evaluation including one at Eastman Kodak. With Dr. Victor Weidner, Dr. Mavrodineanu produced fluorescent sheets of polytetrafluoroethylene (Teflon) doped with inorganic phosphors by sintering a mixture of the polymeric material with the phosphors at 370°. These sheets, 2 mm in thickness, were cut into pieces and mounted in brass holders which fit into standard 1 x 1 cm cuvette chambers. By mounting the fluorescence material at a 60° angle to the excitation beam of spectrofluorometers, one can record the fluorescence with reduced interference from scattered light. We evaluated four fluorescent sheets (blue, green, yellow, and orange) which were provided in a set with one blank polytetrafluoroethylene sheet as a blank. For these samples, we evaluated 1. stability. 2. intensity, 3. spectra, 4. interference from scatter, 5. lifetime: We found: the samples were exceedingly stable, as expected from solid state inorganic phosphors. Leaving the samples in the xenon arc beam for 8 hours, caused no bleaching, nor did the concomitant rise in sample temperature. The intensity of the samples was found to be adequate, in that slits of different sizes plus excitation at various wavelengths resulted in sufficient fluorescence to be measured easily. In measuring the fluorescence emission spectra, we



encountered interference from scattered and stray light. We could sometimes correct for this parasitic light by running a blank with the undoped sheet. However, some warping in the sheets caused an apparent wavelength shift when blank and samples were being compared. Unwanted light could be eliminated by some suitable yellow cutoff filters for all samples except that emitting in the blue. The emission spectra of all four samples could be obtained after correction with blank and/or cutoff filtration. We concluded that the general principle of using solid, inorganic phosphors as fluorescence standards is a promising approach due to the stability and spectral region of emission. However, the particular samples studied need improvement especially in making the sample thickness and mounting identical to that of the blank. In addition, the lifetimes of the phosphors was in the micro to millisecond range and therefore not useful for lifetime standards for solution organic chemists. A preliminary report of these findings with enclosed spectra was sent to Dr. Mavrodineanu, and it is possible that a joint communication will be forthcoming, and that improved samples will be produced as a result of our study.

2. The method for measuring the association of metal ions to peptides containing tryptophan or tyrosine has been completed and published. It had been found that  $\text{Cu}^{++}$  and  $\text{Ni}^{++}$  cause quenching of the fluorescence of tryptophan or tyrosine when complexes are formed in solution. The quenching can be used to determine the association constants. The constants obtained for the amino acids, as well as di- and tri- peptides containing tryptophan or tyrosine agreed well with literature values.

#### Proposed Course:

We will encourage the National Bureau of Standards to improve their fluorescence standards and to find a phosphor extending into the ultraviolet in the region of protein emission. With Dr. Knutson we plan to set up a microchannel plate detector for better time resolution, and to set up a hybrid pulse and phase shift fluorometer. This will allow us to take advantage of the wide excitation wavelength range available with a phase shift system based on a xenon arc and a Pockel cell, and to show how the harmonic content of the laser can be used in a phase shift system requiring modulated light. We also plan to obtain a pressure cell and obtain data on oxygen quenching or enzyme kinetics at pressures of several kilobars.

#### Publications:

1. Chen, R. F.: Fluorescence Quenching as a Parameter for Measuring Complex Formation Between Metal Ions and Aromatic Amino Acids and Peptides, Analytical Lett. 19(9-10), 663-677, 1986.





# NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01413-25 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Biophysical Methods for Study of Bio-Macromolecular Reactions

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. R. L. Berger Chief, Biophysical Instrumentation Section LTD:NHLBI

## COOPERATING UNITS (if any)

NIA, Lab. Molecular Biology (J. Froehlich), Univ. of Pennsylvania ( L. Thiebault), Biomedical Engineering & Instrumentation Branch (H. Casio), Commonwealth Technology, Alexandria, VA, Div. Blood Resources, LAIR (R. Winslow).

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

Biophysical Instrumentation Section

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The adaptation of the isoionic hemoglobin preparation developed in this Laboratory by the joint Forces Casualty Care Blood Resources Division based at Letterman Army Research Institute has provided stimulus to a renewed interest in several instruments developed in this laboratory. A modified oxygen binding to hemoglobin apparatus has been set up and is presently undergoing tests in the Laboratory of Molecular Biology, NIA. A highly modified cell is nearing completion which will permit the measurement of the oxygen dissociation curve of hemoglobin simultaneously by a manometric method and spectroscopy. Such data are needed if the various models of how hemoglobin binds oxygen are to be critically tested. In collaboration with computational scientists at UCLA and DCRT a considerable effort has been mounted to have all of the needed programs operating on the local IBM AT rather than a main frame computer.

The inertial drive quench flow apparatus and thermal-optical stopped flow apparatus have had a number of improvements made to them over the last year and it is expected that the quench flow machine will go operational in the laboratory of Biological Chemistry, NIA by the end of the summer. It has become clear that for the thermal stopped flow to be useful a differential version will need to be built and this should be finished in the next year. Experiments are planned on the ATPase ATP reactions of muscle as a test of the system.

848



## Project Description

### Objectives:

The major objective of the work of this section is to develop new instruments, analysis methods, and data handling techniques for the physiochemical study of biological reactions.

### Methods Employed:

Biological physics attempts to find the simplest explanation for complex systems. This section is particularly interested in model reactions because it is believed that considerable progress can be made in developing a more general theory of structure-function regulation if these reactions are understood in detail. The reaction of hemoglobin with various ligands and effector molecules (small molecules which alter the structural properties of the large protein molecule) is used as the principle model of a regulatory protein reaction. The methods used in the investigation of the mechanisms of enzyme action are primarily those of pre-steady state chemical kinetics and thermodynamics. Measurements of the appropriate parameters are made by developing the necessary equipment to mix solutions rapidly and follow the course of resulting chemical reaction by optical, thermal, pH electrode, etc. detectors.

### Major Findings:

The adaptation of the isoionic hemoglobin preparation developed in the laboratory by the joint forces Casualty Care Blood Resources Division based at Letterman Army Research Institute has provided stimulus to a renewed interest in several instruments developed in this laboratory. A modified oxygen binding to hemoglobin apparatus has been set up and is presently undergoing tests in the Laboratory of Molecular Biology, NIA. A highly modified cell is nearing completion which will permit the measurement of the oxygen dissociation curve of hemoglobin simultaneously by a manometric method and spectroscopy. Such data are needed if the various models of how hemoglobin binds oxygen are to be critically tested. In collaboration with computational scientists at UCLA and DCRT a considerable effort has been mounted to have all of the needed programs operating on the local IBM AT rather than a main frame computer.

The inertial drive quench flow apparatus and thermal-optical stopped flow apparatus have had a number of improvements made to them over the last year and it is expected that the quench flow machine will go operational in the laboratory of Biological Chemistry, NIA by the end of the summer. It has become clear that for the thermal stopped flow to be useful a differential



version will need to be built and this should be finished in the next year. Experiments are planned on the ATPase and ATP reactions of muscle as a test of the system.

Significance to Biomedical Research and the Program of the Institute:

Molecular biology and biological physics study the underlying physical and chemical processes involved in biological reactions at the cellular level. This section concentrates on developing new methods that will permit careful measurement of these reactions so that theories of the reaction mechanism can be critically tested.

Publications

None.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01414-15 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Biocalorimeters for Solution and Cell Biochemical Studies

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.	R. L. Berger	Chief, Biophysical Instrumentation Section	LTD:NHLBI
	T. Kolobow	Medical Officer	LTD:NHLBI

## COOPERATING UNITS (if any)

C. Mudd, Biomedical Eng. & Instrum. Branch, DRS, Richard Shrager, DCRT, M. Marini, Division of Blood Resources, LAIR, N. Davids, Penn State Univ., Commonwealth Scientific, Alexandria, VA, and Commonwealth Technology, Inc., Alexandria, VA., K. Breslauer, Dept. of Chemistry, Rutgers Univ.

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

Biophysical Instrumentation Section

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The development of a new all tantalum stopped flow microcalorimeter has proceeded to the point that a complete set of test data have been run and the instrument is now operational. Experiments are in progress on a number of DNA drug binding reactions. This work is being done in collaboration with scientists at Rutgers University. Only 80 microliters of each reagent are needed per reaction. The sensitivity is ten times that of the batch calorimeter requiring 1 ml of each reagent. For reactions with halflives faster than 0.1 second, a reaction can run every 3 minutes. The instrument is controlled by a microcomputer which takes the data and analyzes it on line. Extensive work with hemoglobin is planned for the coming year in collaboration with scientists at NIA, LAIR and Johns Hopkins University.

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Project Description

The objectives of this project are to develop methods of measuring the enthalpy of both solution and cell reactions. This project is also aimed at utilizing the calorimeter as a modern Warburg respirometer with a thousand-fold increase in sensitivity.

Methods Employed:

Utilizing various biochemical and cellular reactions as model systems, appropriate instrumentation is designed and built in this laboratory, where close interaction between investigator and instrument developer can be carried out.

Major Findings:

The development of a new all tantalum stopped flow microcalorimeter has proceeded to the point that a complete set of test data have been run and the instrument is now operational. Experiments are in progress on a number of DNA drug binding reactions. This work is being done in collaboration with scientists at Rutgers University. Only 80 microliters of each reagent are needed per reaction. The sensitivity is ten times that of the batch calorimeter requiring 1 ml of each reagent. For reactions with halflives faster than 0.1 second, a reaction can run every 3 minutes. The instrument is controlled by a microcomputer which takes the data and analyzes it on line. Extensive work with hemoglobin is planned in the coming year in collaboration with scientists at NIA, LAIR and Johns Hopkins University.

Publications:

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01421-12 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Electrochemical and Physiological Methods for Cell Research

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.	R. E. Steele	Physical Science Investigator	LTD:NHLBI
Others	R.L. Bowman	Chief, LTD	LTD:NHLBI
	H. S. Kruth	Senior Investigator	EA:NHLBI
	J. W. Handler	Section Chief	KE:NHLBI
	A. S. Preston	Chemist	KE:NHLBI
	J. P. Johnson	Chief, Nephrology	WRAIR

## COOPERATING UNITS (# any)

Laboratory of Experimental Atherosclerosis, NHLBI  
Laboratory of Kidney and Electrolyte Metabolism, NHLBI  
Department of Nephrology, Walter Reed Army Institute of Research

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The porous bottom culture dishes (PBCDs) developed in this laboratory for the study of epithelial cells grown in confluent layers are now being used in more than 500 laboratories world-wide. This large utilization has resulted in part from the fact that PBCD are now commercially available from 2 sources (Millipore Corp. and Costar Corp.) with several types of porous membranes with various cell culture treatments and coatings. Many different types of epithelial and some endothelial and smooth muscle cells are being studied using the PBCDs. The objectives of the research projects being undertaken with the PBCDs go far beyond anything we had in mind at the outset of our development of them.

The PBCDs made with collagen membranes also made in this laboratory still provide the best optical properties (for phase microscopy) of all the "homemade" and commercial PBCDs. Many cell types grow well on the collagen also. In fact, endothelial cells have been grown on one side of the collagen membrane and smooth muscle cells on the other side. This makes a good model of a blood vessel.

The devices developed in this laboratory to sterilly study sodium transport by confluent layers of epithelial cells grown on the PBCDs are being used in many laboratories. These are now being used for indications of confluency of layers of other cell types.

The major role played by Ca in the regulation of many cellular processes has prompted us to improve methods for measuring free Ca activity in cell systems. We have developed a 2 mm diameter electrode using a hydrophobic porous membrane and neutral carrier Ca exchanger which has a response time of 10 seconds and a resistance of 10 megohms. It is useful for measuring small quantities of solutions representative of cell interiors for many biochemical studies. These electrodes have also been useful in measuring interesting  $\text{Ca}^{++}$  activity reductions in perfusion solutions which apparently result from ion pairing of calcium and bicarbonate ions.

F53



Project DescriptionObjectives:

- (1) Develop instruments and methods to make possible the study of cells in culture in ways that have not been done before.
- (2) Improve methods for the measurement of  $\text{Ca}^{++}$  activity in and around cells.

Measurements of epithelial and endothelial cells in culture are our prime objectives.

Methods Employed and Major Findings:

The porous bottom culture dishes (PBCDs) developed in this laboratory for the study of epithelial cells grown in confluent layers are now being used in more than 500 laboratories world-wide. This large utilization has resulted in part from the fact that PBCD are now commercially available from 2 sources (Millipore Corp. and Costar Corp.) with several types of porous membranes with various cell culture treatments and coatings. Many different types of epithelial and some endothelial and smooth muscle cells are being studied using the PBCDs. The objectives of the research projects being undertaken with the PBCDs go far beyond anything we had in mind at the outset of our development.

The PBCDs made with collagen membranes also made in this laboratory, still provide the best optical properties (for phase microscopy) of all the "homemade" and commercial PBCDs. However, the commercial PBCDs are appearing with increasingly good optical properties. The collagen membrane PBCDs have been very useful for growing endothelial and smooth muscle cells. In fact endothelial cells have been grown on one side of the collagen membrane and smooth muscle cells on the other side. This makes a good model of a blood vessel.

The devices developed in this laboratory to sterily study sodium transport by confluent layers of epithelial cells grown on the PBCDs are being used in many laboratories. As work with endothelial and other nonepithelial cells has increased, interest in these devices has developed for the measurement of electrical resistance of the cell layers as an indication of confluency or permeability to ions.

The fact that many cells in vivo grow on basement membranes which have permeabilities that are significantly smaller than those of the membranes used on the PBCDs in the past, has caused us to begin an investigation of reconstituted cellulose membranes which are often used for dialysis. Experiments spread over several years have shown that cell attachment to such membranes requires some sort of surface treatment. One treatment that gives cell attachment for long periods in the brief exposure of the surface of the



dialysis membrane to a methane-air flame. The optical properties of the resulting PBCDs are even better than the PBCDs made with "homemade" collagen membranes.

The major role played by Ca in the regulation of many cellular processes has prompted us to improve methods for measuring free Ca activity in cell systems. We have developed a small electrode using a hydrophobic porous membrane and neutral carrier Ca exchanger which has a fast response and low resistance. It is useful for measuring small quantities of solutions representative of cell interiors for many biochemical studies. These electrodes have also been useful in measuring interesting  $\text{Ca}^{++}$  activity reductions in perfusion solutions which apparently result from ion pairing of calcium and bicarbonate ions. The electrodes are made from 60 mm lengths of 2 mm o.d. Pyrex glass tubing by cementing a piece of porous polypropylene membrane on one end with medical grade silicone rubber cement. After polymerization of the rubber the pores of the membrane are filled with a hydrophobic  $\text{Ca}^{++}$  neutral carrier mixture developed by Simon's group in Switzerland. This is done by touching the membrane to a small drop of the mixture on a piece of glass. The resulting electrodes have several desirable characteristics: 10 sec. response time, 10 megohm resistance, 2 week lifetime and quickly renewable with negligible equilibration time required. With these characteristics the electrodes should work with most existing pH meters. A small flow cell has been fitted to the electrode with the aid of an O ring. This cell and tubing has a filling volume of 50 microliters. Many solutions are made up for biochemical studies based on calculations using a variety of calcium buffer systems (i.e. EGTA). Actually measuring the resulting value with an electrode similar to this appears important just as it is when pH buffered solutions are made.

#### Publications:

Steele, R. E., Preston, A. S, Johnson, J. P., and Handler, J. S.: Porous-bottom dishes for culture of polarized cells. Am. J. Physiol. 251 (Cell Physiol. 20): C136-C139, 1986.

Handler, J. S., Green, N., and Steele, R. E.: Porous bottom culture dishes for studying transport and differentiation, Methods in Enzymology, in press.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01452-04 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Time Resolved Fluorescence Spectroscopy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.	J. R. Knutson	Sr. Staff Fellow	LTD:NHLBI
Others	R. F. Chen	Sr. Investigator	LTD:NHLBI
	J. L. White	Engineer	BEIB:DRS
	Y. L. Ong	Intern	LTD:NHLBI

COOPERATING UNITS (if any) P. Hensley (Georgetown); A. Ginsburg (LB:NHLBI); M. Han, L. Brand, D. Walbridge, S. Roseman and C. Anfinsen (Johns Hopkins Univ.); C. N. Rafferty (WRAIR); L. Davenport and D. Goss (CUNY); P. Neyroz (U. Diparma); A. Russo (NCI); B. Taffe (JHMI).

## LAB/BRANCH

Laboratory of Technical development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.25

## PROFESSIONAL:

1.25

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A new time-resolved fluorescence spectrophotometer was developed to provide rapid collection and analysis of macromolecular size, decay lifetimes, and spectra. The instrument was exploited to study protein associations (eg; conformational changes in OTCase and arginase with P. Hensley, Georgetown; enzyme I dimerization with M. Han, L. Brand, S. Roseman, JHU; decays of glutamine syntetase with A. Ginsburg, H/LB; eu.if.2 with D. Goss, CUNY). The system was modified to speed studies of conformational change in proteins (eg; thiroedoxin sulfhydryl reduction/oxidation and folding, with M. Han, C. Anfinsen, L. Brand, JHU; rapid denaturation of HLADH monitored by lifetime and rotation changes. Model tryptophyl-related systems (melatonin, serotonin, trp peptides, copper and nickel quenchings) were examined with Dr. Chen.

"Global analysis" methods were developed to quantitate macromolecule axial ratios, to study proteins and lipids exhibiting distributed decay, and to link spectral signatures to conformations.

A variety of other biochemical applications -

Aggregation and self quenching of solutes inside liposomes (R. Chen H:LTD).

Sulfhydryl probes for oxidation defense profiles (A Russo C:ROB).

Tryptophyl fluorescence changes accompanying oxidative damage of enzymes (B. Taffe JHU, R. Levine H:LB).

DNA carcinogen adduct detection (D. Manchester C:LMC), Microwave effects on liposome structure (C Rafferty WRAIR).

Structural fluctuations in lipid bilayers ( L. Davenport CUNY).

Several other new laser fluorescence instruments were designed and are being prototyped continuously.



Project DescriptionObjectives:

We wish to develop instrumental approaches to clarify time-resolved fluorescent signals from proteins and membrane structures. This will help us to untangle the complex emissions that biological systems display. Once these systems are broken down into components, it becomes possible to associate spectral features with reporting groups that represent particular sizes and/or conformations. Binding events (and related biological events) can be examined as they perturb associated spectra. Fluorescence spectra and decays are exquisitely sensitive indicators of structure and dynamics. The only bar to their effective use is the difficult task of "untangling" complex, mixed signals. The state-of-the-art instruments we have developed, when combined with our recent data analysis schemes, greatly facilitate the recovery of multiple signals from complex systems. These resolved components help us understand the basic function of proteins and lipid bilayer membranes.

Major Findings:

The laser-based fluorometer recently developed in LTD was exploited to study a variety of structure/dynamics questions in proteins and membranes. Since many system capabilities are new, we presented "demonstrations" of its more unique features. In particular, we focussed attention this year on fluorescence lifetime (nanosecond decay) measurements during protein folding/unfolding reactions. We combined our instrument's ability to collect precise data in a few seconds with our new "global analysis" programs to separate the distinct stages in unfolding. We published accounts of HLADH acid denaturation (1,2,3), thioredoxin sulfhydryl oxidation/reduction (4,5,6) and enzyme I dimerization (7,8). These systems were studied in collaboration with the laboratories of L. Brand, C. Anfinsen and S. Roseman at JHU. In addition, we have just begun collaborations with R. Levine (NHLBI:LB) and B. Taffe (JHMI) to apply these methods to oxidative protein damage. Recently, we added the ability (T-format) to examine residue rotations that accompany such changes. We are modifying the instrument to achieve "stopped-flow" mixing speeds.

Since earlier instruments often required an hour to obtain a single decay profile, our work in seconds represents a significant advance. Our new multichannel photomultiplier is being adapted now to provide four or more decay curves (at different wavelengths) simultaneously. Meanwhile, our emission scanning capability has been exploited. For example, the unique Trp in OTCase provides three signals (DAS-decay associated spectra) having different response to single vs. bisubstrate binding conformations, along with changes in quenching rates (9). We have also examined the ligand-dependent decays of enzyme I; thioredoxin, eukaryotic initiation



factor 2, arginase and glutamine synthetase. We published a new (global) method to obtain protein axial ratios (10). Continuing the process of "global" software development, we demonstrated the inability of ordinary "lifetime distribution" analyses to distinguish discrete vs distributed decay, then provided a global alternative that can discriminate between models (17). Discrete vs. continuous lifetime structure is a key debate in protein fluorescence at this time, and this new tool should help dispel confusion. Harmonic (UV) autotracking was added to the laser this year, so excitation scans become possible as another tool in our protein dynamics kit.

A second major area of investigation is membrane structure. We have developed a fluorescence probe (coronene) which is uniquely sensitive to lipid fluctuations (gel-fluid equilibrium). These structural fluctuations, inferred from other work, have been implicated in a variety of key membrane functions (eg, channel gating, membrane bound enzyme/receptor activation, electrolyte transport). These important fluctuations have not been directly observable until now. With our collaborators ( L. Brand of JHU and L. Davenport of (CUNY) we have expanded the search for similar probes while we characterized coronene in different membrane types (11, 12). With Dr. Chen, we examined the mechanism of remanent fluorescence for self quenched, lipid encapsulated chromophores ((13); see also Dr. Chen's report). We also used this type of assay to debunk previous experiments that had reported anomalous permeability changes in microwave-irradiated liposomes (14). This work extended our collaboration with C. Rafferty (WRAIR) discussed in last year's report. We published associative/global approaches to several other lipid probe systems (15, 16).

In addition to the lipid and protein studies, we began several other collaborative efforts. With A. Russo (C:ROB) and P. Smith (DRS:BEIB), we began to develop luminescence dosimetry for photodynamic tumor therapy. Current efforts include assessment of fluorescent sulfhydryl (esp. glutathione) probes to examine oxidation protection mechanisms in the cell. Most recently, we have entered collaborations with D. Manchester (C:LMC), to enhance the detectability of DNA-carcinogen adducts and with V. Mangianello (H:LMB), to exploit the fluorescence of cilostamide, a specific PDE inhibitor.

In all, the expansion/upgrading of our fluorometer has proceeded concurrently with its use. It is a unique resource, although other facilities worldwide are now being changed to incorporate more of its design features.



Publications

1. Walbridge, D. G., Knutson, J. R., and Brand, L.: Nanosecond time-resolved fluorescence measurements during protein denaturation. *Anal. Biochem.* 161:467-478, 1987.
2. Walbridge, D., Knutson, R. J., Han, M. and Brand, L.: *Biophys. J.* 51:, 284a.
3. Han, M. K., Walbridge, D. G., Knutson, J. R., and Brand, L.: Enhancement of time-resolved fluorescence spectroscopy by overdetermination, in *Fluorescence Biomolecules*, Plenum, in press, 1987.
4. Han, M. K., vandeVen, Walbridge, D., Knutson, J. R., Lessick, R., Anfinson, C., and Brand, L.: Nanosecond time-resolved fluorescence of thioredoxin, in *Fluorescence Biomolecules*, Plenum, in press, 1987.
5. Han, M. K., Walbridge, D. G., Knutson, J. R., Brand, L., and Roseman, S.: Nanosecond time-resolved fluorescence kinetic studies of the 5,5'-dithiobis(2-nitrobenzoic acid) reaction with Enzyme I of the phosphoenolpyruvate:glycose phosphotransferase system. *Anal Biochem.* 161:467-478, 1987.
6. VandeVen, M., Han, M., Walbridge, D., Knutson, J. R., Shin, D., Anfinson, C., and Brand, L.: *Biophys. J.* 51:275a.
7. Han, M., Walbridge, D., Knutson, J. R., Hong, S., Roseman, S., and Brand, L.: *Biophys. J.* 51:276a.
8. Han, M. K., Walbridge, D. G. Knutson, J. R., Brand, L.: Nanosecond time-resolved fluorescence: Kinetic studies of macromolecules. *Proceedings of International Biophysics Congress*, in press, 1987.
9. Hensley P., and Knutson, J. R.: *Fed. Proc.* 46:6,2023.
10. Beechem, J. M., Knutson, J. R., and Brand, L.: Global analysis of multiple dye fluorescence anisotropy experiments on proteins. *Biochem. Soc. Trans.* 14:832, 1986.,
11. Davenport, L., Knutson, J. R., and Brand, L.: *Biophys. J.* 51:537a.
12. Davenport, L., Knutson, J. R., and Brand, L.: Studies of membrane heterogeneity using fluorescence associative techniques. *Faraday Discussion*, 81-94, 1986.





13. Chen, R. F., and Knutson, J. R.: Biophys. J. 51:539a.
14. Rafferty, C. N., and Knutson, J. R.: Proc. B.E.M.S., in press.
15. Davenport, L., Knutson, J. R., and Brand, L.: Proc. Membrane Fusion, in press.
16. Davenport, L., Knutson, J. R., and Brand, L.: Subcellular Biochemistry, in press.
17. Knutson, J. R.: Global analysis of fluorescence data: some extensions. Biophys. J. 51:285, 1987.
18. Knutson, J. R., Davenport, L., Beechem, J. M., Walbridge, D. G., and Brand, L.: Associated spectra and the multidimensional nature of fluorescence spectroscopy, in Excited State Probes in Biochemistry and Biology. A. G. Szabo and L. Massotti, eds., Plenum, in press, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01455-03 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Foam Countercurrent Chromatography (CCC)

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. Y. Ito Senior Investigator LTD:NHLBI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Efficient foam separation was achieved by dual CCC through a long multilayer coil mounted on the cross-axis synchronous flow-through coil planet centrifuge (X-axis CPC).

A series of preliminary experiments was performed to investigate various physical factors such as revolutional speed, liquid feed rate, N<sub>2</sub> gas feed pressure, etc., to optimize operational conditions.

Potential capability of the method was demonstrated in separations of two sets of test samples using sodium dodecyl sulfate (SDS) as a foaming agent. Rhodamine B with high foam affinity was quickly separated from Evans blue lacking foam affinity. A high concentration of salts such as Na<sub>2</sub>HPO<sub>4</sub> in the surfactant solution effectively lowered the foam affinity of basic dyes, thus yielding complete peak resolution of two basic dyes, rhodamine B and methylene blue, according to a subtle difference in foam affinity.

fb1



Project DescriptionObjectives:

Foam CCC with the cross-axis synchronous flow-through coil planet centrifuge (X-axis CPC).

Methods Employed and Major Findings:

A multilayer coil (2.6 mm i.d., 40 m long, and 200 ml capacity) was mounted on a holder (7.5 cm hub diameter) of our prototype X-axis CPC (for the design of the apparatus, see Annual Report Z01 HL 01462-01 LTD). The column is equipped with five flow lines for dual CCC operation as described earlier in Z01 HL 01455-01 LTD.

Various physical factors involved in dual CCC were studied by the aid of a parameter,  $V_F/V_L$  (volume ratio between foam and liquid fractions) which is closely correlated with the amount of liquid retained in the column and also the rate of  $N_2$  that flows through the column. In large  $V_F/V_L$  values such as 1 or greater, liquid volume retained in the column was increased to over 50 ml or 25% of total column capacity accompanied by a reduced  $N_2$  flow which would result in a long retention time with excessive sample band broadening of foam fractions. On the other hand, small  $V_F/V_L$  values like 0.1 or less indicates that the liquid retention volume is reduced below 15 ml or 7.5% of the total column capacity. Although this permits a high flow rate of  $N_2$  through the coil to yield rapid and efficient foam separation, the runs are often complicated by carryover of the foam into the liquid fraction causing contamination. Optimum range for  $V_F/V_L$  was found between 0.2 and 0.6 which yielded liquid retention of 20 ml (10%) and 40 ml (20%), respectively. The above condition was achieved by manipulating the needle valve on the liquid collection line under a set of operational parameters: liquid feed rate 200 ml/h;  $N_2$  gas feed pressure 120 psi; and resolution 550-600 rpm.

Chemical factors also play an important role in foam CCC. Addition of a small amount of salts in the surfactant solution effectively increases stability of the foam without substantially affecting the foam affinity of samples and prevents adsorption of samples onto the column wall. A high concentration of salts tends to reduce the foam affinity of the samples to various degrees according to their molecular structure, thus providing the basis for separation of two basic dyes, rhodamine B and methylene blue.

Potential capability of the present method was demonstrated on separations of two sets of test samples using sodium dodecyl sulfate (SDS) as a foaming agent. In each separation the column was rotated at 550 rpm while the SDS



surfactant solution and  $N_2$  gas were simultaneously introduced into the rotating column through the respective feed lines. After steady state dual countercurrent equilibrium was reached, sample solution (0.5 ml) was injected into the middle portion of the column through the sample feed line. Effluents from the foam and liquid collection lines were each separately fractionated at 30 second intervals for absorbance determination with a Zeiss spectrophotometer.

Separation of rhodamine B (basic dye) and Evans blue (acid dye) was performed with 1mM SDS containing NaCl at 5mM concentration. Because of the negative electric charge of the SDS molecule, positively charged rhodamine B had affinity to the foam and quickly eluted through the foam collection line whereas negatively charged Evans blue was carried with the liquid stream in the opposite direction and eluted through the liquid collection line.

The experiments were continued to explore the possibility of separating two basic dyes, rhodamine B and methylene blue, by adding a large quantity of salt in the surfactant solution. A high concentration of  $NaH_2PO_4$  at 0.5 M in 0.5 mM SDS solution selectively lowered foam affinity of rhodamine B relative to that of methylene blue. Consequently, these two dyes were completely separated according to a subtle difference in foam affinity and eluted out through the foam collection line.

#### Proposed Course:

The present methods will be applied to more useful biological samples such as macromolecules and cells, with or without surfactant. Nontoxic foaming agent such as bovine serum albumin may be used for collecting cell particles.

#### Publication:

Ito, Y.: Foam countercurrent chromatography with the cross-axis synchronous flow-through coil planet centrifuge. J. Chromatogr., in press.

#### Patent:

Ito, Y.: U. S. patent 4,615,805: Foam Countercurrent Chromatography Based on Dual Countercurrent System, Oct. 7, 1986.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01459-02 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of Unilateral Distribution of Two Solvent Phases in the Rotating Coil

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. Y. Ito Senior Investigator LTD:NHLBI

## COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, NIH

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.7

## PROFESSIONAL:

0.5

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A simple rotary device was fabricated to study motion and distribution of two immiscible solvent phases in a rotating coil. Experiments were performed by glass coils of 1 to 2 cm i.d., with helical diameters ranging from 2.5 cm to 20 cm. The results obtained with various two-phase solvent pairs are summarized below on the basis of interplay between the two major physical factors, i.e., solvent-wall interaction and Archimedean screw force.

1. Effect of solvent-wall interaction: High interfacial tension of some binary solvent systems causes plug of phase segments in a narrow-bore coil to interfere with movement of the two phases in the rotating coil.

2. Combined Effects: In this transitional case, one phase with wall-surface affinity passes through segments of the other phase affected by Archimedean screw force and unilaterally distributed in the head side of the coil.

3. Archimedean screw effect: In the rest of the cases, two phases display characteristic distribution consisting of 4 stages according to the applied rotational speed.

Stage I: Slow coil rotation distributes two phases evenly in the coil.

Stage II: At critical rotational speed, two phases form unilateral distribution. Except for chloroform/water, the heavier phase occupies the head side of the coil.

Stage III: Unilateral distribution returns to even distribution through a variety of distribution patterns.

Stage IV: Finally, high rotational speed establishes hydrostatic phase distribution.

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## Methods Employed and Major Findings:

### 1. Apparatus:

A simple rotary coil assembly was fabricated for the present study. The apparatus horizontally holds a cylindrical coil holder which is rotated about its own axis at various speeds from 0 to 500 rpm by a motor equipped with a speed control unit. The coil holder is easily interchangeable so as to mount coils with different helical diameters ranging from 2.5 cm to 20 cm. Each coil was made from 1 cm or 2 cm i.d. glass tubing with ca 1 mm wall thickness and equipped with a glass stopper at each terminal.

### 2. Procedure:

Experiments were performed with nine volatile two-phase solvent systems with a broad range of hydrophobicity. Before starting experiment each pair of solvents was thoroughly equilibrated in a separatory funnel at room temperature. In order to facilitate observation, a small amount of dye was added to color one of the phases. Sudan III was conveniently used to color the nonaqueous phase of all solvent pairs except for the hexane/methanol in which acid fuchsin was used to color the aqueous phase.

In each experiment, the coil was first entirely filled with about equal volumes of the two solvent phases and both ends were sealed with stoppers. Then, the coil was rotated at the lowest speed of 10 rpm. After a steady state hydrodynamic equilibrium was reached, the rotation was stopped to allow the two phases to settle in each helical turn. The length of the phase segment occupying each helical turn was measured for several turns on the head side of the coil. The experiment was continued by gradually increasing the rotational speed, until the two phases established a stable hydrostatic distribution by the centrifugal force created by rotation. After all solvent systems were tested with a plain glass coil, the internal wall surface of the coil was siliconized to repeat the same series of experiments to study the effect of solvent-wall interaction on the phase distribution. Using a set of experimental data obtained above, the phase distribution diagram was produced by plotting percentage volume distribution of the heavier phase against the applied rotational speed for each solvent pair in each glass coil.

### 3. Results:

The overall results of the present studies may be summarized on the basis of two physical factors which play a major role in hydrodynamic phase distribution in the rotating coil, i.e., solvent-wall interaction and Archimedean screw force:



a) High solvent-wall interaction: Movement of a highly hydrophobic binary solvent pair typically hexane/water is largely interfered by strong solvent-wall interaction in the narrow-bore coil. In this case, segments of one of the phases with less wall surface affinity plug the opening of the coil and solvent film is very sluggish or completely interrupted unless application of a high rotational speed produces a strong centrifugal force field which establishes hydrostatic phase distribution in the coil.

b) Combined effects: In this transitional case, two-phase flow induced by the Archimedian screw force is largely affected by solvent-wall interaction to form completely reversed uni-lateral phase distribution in the untreated and siliconized coil, i.e., the head phase is the aqueous phase in the untreated coil while it becomes the nonaqueous phase if the same coil is siliconized. This phenomenon was observed in few cases: chloroform/water in the 1 cm i.d. coil with 5 cm helical diameter and hexane/water in the 2 cm i.d. coil with 10 cm helical diameter.

c) Archimedean screw effect: In the rest of the cases where the effect of solvent-wall interaction is insignificant, the Archimedean screw force plays a major role in phase distribution in the rotating coil. Under these circumstances, two solvent phases generally display characteristic distribution consisting of four stages: In the slow rotation of 0 to 20 rpm, the two phases distribute fairly evenly in the coil (Stage I). When the rotational speed is increased, the heavier phase tends to occupy more space in the head of the coil and at a critical speed the two phases are completely separated in the coil with the heavier phase on the head side and the lighter phase on the tail side (Stage II). After this critical rpm range, the amount of heavier phase on the head side tends to decrease rather sharply, often crossing below the 50% line (Stage III). Further increase in the rotational speed again yields even distribution of the two solvent phases in the coil apparently due to the centrifugal force effects (Stage IV). In large helical diameter coil, all these stages tend to shift toward the lower rpm range with proportionally reduced width of each stage. One exception was found in hydrodynamic behavior of chloroform/water which exhibited, in Stage II, the completely reversed phase distribution pattern, i.e., the lighter aqueous phase was distributed on the head side and the heavier nonaqueous phase on the tail side. This particular finding may provide an important clue to the mechanism acting in the two-phase distribution in a rotating coil. In addition some interesting variation was observed in Stages II-III where the major peak (Stage II) was followed by a minor peak, forming either a shoulder or a completely resolved peak.

#### Publications:

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01461-02 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hot Tip Catheter for Percutaneous Removal of Atherosclerotic Plaque

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.	R. L. Bowman	Chief, LTD	LTD:NHLBI
	D. Y. Lu	Staff Associate	CB:NHLBI
	M. Leon	Sr. Investigator	CB:NHLBI
	L. G. Prevosti	Special Volunteer	CB:NHLBI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Thermal angioplasty is a newly developed technique that employs a heated metallic tip to thermally ablate atherosclerotic plaques for recanalization of obstructed peripheral arterial blood vessels. However, the current Laser Thermal Probe employs a high power continuous argon laser which is large in size and high in cost. Last year, we developed an electrically heated thermal tip catheter as a possible alternative to the laser probe, and preliminary testing of this device in an in vivo animal model appears promising.

Another economical method of heating a metallic tip is to harvest the chemical energy from the combustion of hydrogen gas. This energy can be released and harvested inside a catheter tip in a safe manner by utilizing a palladium sponge catalyst which initiates and maintains the chemical combustion in a controlled fashion. A prototype catalytic thermal tip catheter has been designed and fabricated for in vitro testing. A temperature feed back control device has been added to avoid excessive tissue heating with the aim of minimizing the incidence of vessel wall perforation. Extensive in vivo testing in an atherosclerotic animal model is planned prior to anticipation of clinical human trials.

f67





Project DescriptionObjectives:

Laser thermal angioplasty (TA) using a metal-tipped quartz fibers has been used successfully to recanalize obstructed human peripheral arteries and preliminary studies are ongoing in patients with coronary artery disease. A new catalytic thermal tip (CTT) catheter has been designed providing an alternative energy source for TA without the expense and technical support needed for a laser operated device.

Methods Employed:

The CTT utilizes the principle of combustion energy from a stoichiometric ratio of oxygen and hydrogen gases catalyzed by a small piece of palladium sponge within the tip. Gas flow regulates CTT temperature (T) which is monitored by a copper-constantan thermocouple inside the metal tip. A 3F prototype CTT was studied in air and saline, alone and with human atherosclerotic aortic segments. Heating was faster in air ( $>350^{\circ}\text{C}$  in  $<1\text{sec}$ ) than saline (T(max) of  $170^{\circ}\text{C}$  in 5 sec,  $t_{1/2}=0.6\text{ sec}$ ), but thermal relaxation was faster in saline ( $t_{1/2}=1.5\text{ sec}$ ) than air ( $t_{1/2}=8\text{ sec}$ ) due to rapid heat convection in saline. In both air and saline, CTT-tissue contact effects were directly related to T at the tip; histologic thermal injury began at  $T>180^{\circ}\text{C}$  but ablation with crater formation, charring, and polymorphous vacuoles did not occur until  $T>325^{\circ}\text{C}$ . Effective tissue ablation in saline required initial vaporization of the saline at the CTT-tissue interface. We conclude that the CTT catheter is safe, inexpensive, and results in efficacious tissue ablation which can be regulated by T feedback monitoring and may be preferable alternative to laser TA.

Major Findings:

It has been reported that balloon angioplasty failures can be reduced by heating the dilated region to coagulate the protein and "weld" the tissue together to reduce restenosis and possibly reduce thrombogeneity of the region. Heating has been accomplished by the use of laser energy conducted via fiber optics to a diffuser to distribute the energy and heat the tissues through the walls of the balloon. We have developed three simpler methods of delivering the heat. We have demonstrated a method of weaving a simple wire heater over the balloon, a simple wire helix inside the balloon that heats the aqueous filling of the balloon to the therapeutic temperature and also demonstrated that we can heat the liquid inside the balloon by a simple peroxide reaction with relatively innocuous reagents.

Patent:

D. Y. Lu and R. L. Bowman: Oxyhydrogen Catalytic Thermal Tip for Angioplasty, Reference Number E-98-87, submitted, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01462-01 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cross-Axis Synchronous Flow-Through Coil Planet Centrifuge

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. Y. Ito Senior Investigator LTD:NHLBI

## COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, NIH (Ronald Seldon)

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.0

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A novel type of coil planet centrifuge is introduced. The apparatus holds a coil holder in such a way that the axis of the holder is positioned perpendicular to and at a fixed distance away from the centrifuge axis. In maintaining the above orientation the holder undergoes synchronous planetary motion, i.e., revolution around the central axis of the centrifuge and rotation about its own axis at the same angular velocity. Mathematical analysis of acceleration generated by this planetary motion revealed a unique distribution pattern of centrifugal force vectors which promises a high performance of the present system in countercurrent chromatography.

Potential capability of the apparatus has been examined with three types of coiled columns all coaxially mounted around the holder. In single-layer coils typical solvent systems display characteristic hydrodynamic distribution which ensures a stable retention of the stationary phase against heavy sample loading in preparative separations. Direct observation of the hydrodynamic motion in the rotating spiral column revealed vigorous mixing of the two solvent phases throughout the area which indicates a high partition efficiency of the present method. Gram-quantity preparative separations were performed in the multilayer coil with two different sets of test samples: Isocratic elution of dinitrophenyl amino acids yielded high partition efficiency of 1,600 theoretical plates while versatility of the method was demonstrated on gradient elution of dipeptides including two pairs of sequential isomers.

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## Project Description

### Objectives:

Development of the cross-axis synchronous flow-through coil planet centrifuge (x-axis CPC) for performing countercurrent chromatography.

### Methods Employed and Major Findings:

#### 1. Apparatus

The first prototype of the x-axis CPC was constructed at the NIH machine shop. The design of the apparatus is as follows: The motor drives the rotary frame around the central axis of the centrifuge via a pair of toothed pulleys coupled with a toothed belt,. The rotary frame consists of a pair of side-plates rigidly bridged with links to support a column holder and a counterweight holder horizontally in the symmetrical positions at a distance of 10 cm from the central axis of the centrifuge. At the lower position of the side-plates, a pair of countershafts is radially mounted in the symmetrical positions through ball bearings. The stationary miter gear (45°) mounted on the bottom plate of the centrifuge coaxially around the central shaft is coupled to the identical miter gear on the proximal end of each countershaft. This gear coupling results in synchronous rotation of the countershaft on the revolving rotary frame. This motion is further conveyed to the column holder and the counterweight holder by coupling a pair of identical toothed pulleys, one counted on each holder shaft and the other on the distal end of the respective countershaft. Consequently, both the column holder and the counterweight holder undergo a synchronous planetary motion, i.e., rotation about its own axis and revolution around the central axis of the centrifuge at the same angular velocity.

The separation column was prepared by winding a piece of PTFE (polytetrafluoroethylene) tubing (2.6 mm i.d.) directly onto the holder hub making either single layer or multiple coiled layers. The flow tubes from the separation column were first passed through the center hole of the holder shaft and then making a loop enter the side-hole of the central shaft to exit the centrifuge at the top plate where they were tightly held by a clamp. Because of the synchronous planetary motion of the holder, these flow tubes rotate with the rotary frame without twisting, thus providing a rotary-seal-free flow-through elution system for performing countercurrent chromatography.

#### 2. Analysis of Acceleration

Acceleration produced by the X-axis CPC was analyzed mathematically by the aid of a three-dimensional coordinate system. The derived centrifugal force vectors are transformed to the body coordinate system and expressed into a



force distribution diagram using a standard format to facilitate comparative studies with other types of synchronous planetary motion.

The force distribution diagrams produced from the above analysis revealed a unique centrifugal field with three-dimensional fluctuation of force vectors. Coriolis force components are completely separated from the major force field, acting along the axis of the holder. Vigorous oscillation of the above Coriolis force at all locations of the holder strongly indicates high partition efficiency of the present system in performing countercurrent chromatography.

### 3. Phase Retention Studies

Experiments were performed with a short coil prepared from a 2.6 mm i.d. PTFE tube coaxially mounted on a coil holder with three different hub diameters of 5, 10 and 15 cm. Nine volatile two-phase solvent systems with a broad spectrum of hydrophobicity were selected for study. In each test run, the coil was first completely filled with stationary phase. Then, mobile phase was eluted through the coil at a flow rate of 120 ml/h while the apparatus was run at a desired speed of 200, 400, 600, or 800 rpm. From the volume of the stationary phase eluted out from the coil, the percentage retention relative to the total coil capacity was computed to draw a phase distribution diagram. The results revealed a characteristic retention profile which indicates stable hydrodynamic trends of the solvent phases in the present system. Except for high interfacial tension binary systems, all useful solvent systems examined yielded fair retention and exhibited uniform hydrodynamic distribution where the heavier phase is distributed toward the head and the lighter phase, toward the tail.

The above finding clearly indicates great advantage of the present system for performing preparative-scale countercurrent chromatography. Introduction of a large amount of solutes to the two-phase solvent system often substantially alters viscosity and interfacial tension of the solvent phases, which, in the conventional high-speed CCC system, may result in the reversed hydrodynamic trend to cause detrimental loss of the stationary phase from the column. A stable hydrodynamic trend of the present system largely eliminates the above complication to ensure reliable results in large-scale preparative separations.

### 4. Stroboscopic Observation

Distribution and motion of two-solvent phases in the rotating column was observed under stroboscopic illumination. Visibility of the solvent phases was improved by coloring the mobile phase and using a flat single-layer spiral column equipped with a transparent front cover. Each experiment was





initiated by filling the column with stationary phase. Then, the apparatus was rotated at 800 rpm while the mobile phase was pumped into the column at a 120 ml/h flow rate in a proper elution mode.

Stroboscopic observation at the steady state hydrodynamic equilibrium revealed clear evidence of vigorous agitation of the two solvent phases throughout the spiral column but especially at the vicinity of the center of the revolution. This finding promises high performance of the present system in that solutes are continuously subjected to an efficient partition process between the mobile and stationary phases at all portions of the separation column.

#### 5. Preparative-Scale Separations of Test Samples

Potential capability of the present system was demonstrated in chromatographic separations of two sets of test samples in gram quantity using a multilayer coil. The column was prepared from a long continuous piece of 2.6 mm i.d. PTFE tubing by winding it around a spool-shaped holder with 10 cm hub diameter making multiple coiled layers. The total capacity of the coil measured 400 ml. In each separation the column was first entirely filled with stationary phase followed by injection of sample solution through the sample port. Then the mobile phase was introduced into the column at a flow rate of 120 ml/h while the apparatus was run at 800 rpm. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S at 280 nm and fractionated with an LKB fraction collector.

In the first series of experiments a DNP (dinotrophenyl) amino acid mixture was isocratically separated on a two-phase solvent system composed of chloroform/acetic acid/0.1N HCl (2:2:1) with sample size ranging from 100 mg to 2 g. High partition efficiency of 1,600 theoretical plates was noted for 100 mg sample size. Increase of sample size showed little change in peak resolution up to 1g dose, and even at 2 g sample size all peaks were well separated from each other.

In the second series of experiments, dipeptide mixture was separated by gradient elution of dichloroacetic acid concentration (0.01 → 0) in n-butanol/0.1M ammonium formate (1:1). Seven dipeptides were well resolved up to 500 mg sample size.

#### Proposed Course:

Construction of the X-axis CPC with 20 cm revolutionary radius to evaluate its large preparative capacity.



Publications:

1. Ito, Y: Cross-Axis Synchronous Flow-through Coil Planet Centrifuge Free of Rotary Seals for Preparative Countercurrent Chromatography, Part I: Apparatus and Analysis of Acceleration. Sep. Sci. and Tech., in press.
2. Ito, Y.: Cross-Axis Synchronous Flow-through Coil Planet Centrifuge Free of Rotary Seals for Preparative Countercurrent Chromatography. Part II. Studies on Phase Distribution and Partition Efficiency in Coaxial Coils. Sep. Sci. and Tech., in press.

Patent:

Ito, Y.: U. S. Patent pending: Cross-Axis Synchronous Flow-through Coil Planet Centrifuge Free of Rotary Seals: Apparatus and Method for Performing Countercurrent Chromatography.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01463-01 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analytic High-speed Countercurrent Chromatography with a Coil Planet Centrifuge

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.	Y. Ito	Senior Investigator	LTD:NHLBI
	Y. W. Lee	Senior Investigator	Research Triangle Institute

## COOPERATING UNITS (if any)

Research Triangle Institute, NC; Biomedical Engineering and Instrumentation Branch, NIH (Jimmie Powell).

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.3

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A compact table top model of a coil planet centrifuge was constructed for performing analytical countercurrent chromatography. The apparatus holds a multilayer coil (0.85 mm i.d., 70 m long, 38 ml capacity) and a counterweight in the symmetrical positions at 5 cm from the central axis. The maximum revolutionary speed measured 2,000 rpm.

Capability of the apparatus was demonstrated in separation of four indole plant hormones with a two-phase solvent system composed of n-hexane/ethyl acetate/methanol/water (3:7:5:5). Separation was completed within 90 minutes yielding high partition efficiencies ranging from 1,000 to 1,300 theoretical plates. The method may be useful for interfacing to mass spectrometry.

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Project DescriptionObjectives:

Development of Analytical High-Speed Countercurrent Chromatograph.

Methods Employed and Major Findings:I. Apparatus:

A compact table top model of the coil planet centrifuge was designed for the present study. The motor directly drives the rotary frame around the central axis of the centrifuge. The rotary frame consists of a pair of aluminum plates rigidly bridged by links and holds a column holder and the counterweight holder in the symmetrical positions at 5 cm from the central axis of the centrifuge. The holder shaft is equipped with a plastic planetary gear which is coupled to an identical stationary sun gear rigidly mounted on the central axis of the centrifuge. This gear coupling produces a desired synchronous planetary motion of the column holder: The holder revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity. This particular type of planetary motion permits the flow tubes to rotate around the central axis of the centrifuge without twisting, thus facilitating continuous elution of the mobile phase through the rotating column. The revolutional speed of the centrifuge was continuously adjustable up to the maximum rate of 2,000 rpm with a control unit.

Two types of coiled columns were prepared each from a 0.85 mm i.d. PTFE (polytetrafluoroethylene) tube: single-layer coils for phase retention studies and a multilayer coil for analytical separations. The single-layer coil was made from a 5 m length of tubing by winding it tightly around the holder hub of two different diameters, 5 cm and 7.5 cm. The multilayer coil was prepared by winding a long piece of tubing onto the holder with a 5 cm hub diameter, making multiple coiled layers. The total column capacity of the multilayer coil measured about 38 ml. Both feed and return flow tubes from each column were first led through the center hole of the holder shaft, and then by making a loop passed through the side-hole of the short coupling pipe to enter the opening of the central stationary pipe. At the exit from the centrifuge the flow tubes were clamped between silicone rubber sheets by a tube support mounted on the centrifuge wall.





## 2. Phase Retention Studies

Retention of the stationary phase in the rotating coil was studied on a set of volatile two-phase solvent systems with a broad spectrum in hydrophobicity. In each experiment, the short coiled column was first filled with stationary phase. Then, mobile phase was eluted through the column while the apparatus was run at a desired revolutionary speed. Experiments were repeated by applying various operational conditions such as mobile phase (upper and lower phases), revolutionary speed 500, 1,000, 1,500, and 2,000 rpm), and flow rate (12, 24, 60 and 120 ml/h) for each helical diameter of the column. Volume percentage retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase eluted from the column and plotted against the applied revolutionary speed to draw the phase retention diagram.

Overall results indicated that except for extremely hydrophilic butanol systems, all solvent systems examined yielded satisfactory retention of the stationary phase at high revolutionary speeds combined with a moderate range of flow rates up to 60 ml/h.

## 3. Analytical Separations

Analytical capability of the present method was demonstrated with the multilayer coil in separations of indole plant hormones (indole-3-acetamide, indole-3-acetic acid, indole-3-butyric acid, and indole-3-acetonitrile) with a two-phase solvent system of n-hexane/ethyl acetate/methanol/water (3:7:5:5). In each run, the column was first entirely filled with the nonaqueous stationary phase which was followed by injection of sample solution (0.8 ml containing each component 250-750  $\mu$ g) through the sample port. Then, the column was rotated at 2,000 rpm and eluted with the aqueous mobile phase at a flow rate of 50 ml/h. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S at 278 nm and fractionated with an LKB fraction collector. Each fraction was diluted with methanol and the absorbance was measured at 280 nm with a Zeiss spectrophotometer.

All components were well resolved in symmetrical peaks and eluted out within 90 minutes with a high partition efficiency ranging from 11,000 to 1,300 theoretical plates. Similar results were also obtained in separations of s-triazine herbicides yielding peak resolution comparable to that obtained with analytical HPLC.



Proposed Course:

1. Separations of various biological samples which produce complications in analytical HPLC.
2. Interfacing the system to a mass spectrometer with a thermospray method.

Publications:

Ito, Y. and Y. W. Lee: Analytical high-speed Countercurrent Chromatography with a Coil Planet Centrifuge. J. Chromatogr. 391: 290-295, 1987.

Ito, Y.: High-speed Countercurrent Chromatography. Nature 325: 419-420, 1987.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 01464-01 LTD

## PERIOD COVERED

October 1, 1986 to September 30, 1987

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

External Counterpulsation with Elastic Recoil

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.	R. L. Bowman	Chief, LTD	LTD:NHLBI
	D: B. Lu	Medical Staff Fellow	CB:NHLBI

## COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

## LAB/BRANCH

Laboratory of Technical Development

## SECTION

## INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.50

## PROFESSIONAL:

.50

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A new method for producing a rapid pressure pulse to the thighs has been shown to produce an increase in the diastolic run off pressure to effect circulatory support similar to that produced by cuff inflation with fluids that require bulky machines and a heavy pressure box.

The novelty is the use of elastic recoil of rubber to obtain a rapid compression and a reexpansion of the rubber by evacuating the space between the rubber and a rigid half tube. The thighs are each enclosed by two tapered half tubes that hold the rubber against the walls by atmospheric pressure. To compress the thighs air is admitted to the evacuated space and the energy stored in the stretched rubber produces rapid compression. R wave keyed with delay produces ear sphygmographic pulses indicating diastolic augmentation of flow with compensatory changes in the pulse wave indicative of effective cardiac support function.

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Project Description:Objectives:

External counterpulsation applied to the legs has previously been shown to effectively increase tissue perfusion. This research seeks to demonstrate a mechanical design to greatly improve the effectiveness, simplify the design, and reduce patient discomfort.

Instead of applying the counterpulsation by suddenly inflating a sac around the leg we release the energy stored in a stretched rubber sheet to accomplish a very rapid compression of the leg. The energy storage stretch is accomplished by atmospheric pressure acting against an evacuated rigid cylindrical space around the leg during the late diastolic and the systolic period prior to the aortic valve closure time. The use of a suction line to withdraw air from the closed space around the rubber sheet limits any pressure applied to one atmosphere. Application of pressure by the relaxation of the rubber provides a compression that is very rapid and increases as the rubber contracts to a smaller tube. Air is the only moving fluid that moves only during the longer energy storage period of the cycle while the sudden release is accomplished by opening a relatively large port in the rigid cylinder to the atmosphere. No rapid flow is required through long tubes and no rigidity of the external chamber is required to obtain a rapid compression. Further the tapered cylindrical shape of the leg causes the rubber to apply greater and earlier pressure to progress up the leg in a "milking" action to aid the proximal projection of the compression pulse.

Major Findings:

Several test systems that were used to determine the properties of the materials of construction, it became evident that natural gum rubber and an acrylic or polycarbonate outer cylinder were the best materials. The construction required heroic effort to stretch the rubber over the outer shell and the size of the outer shell had to accommodate the foot for application to the patient. This added considerable bulk and construction difficulty until Dr. Lu suggested the possibility that it might work if bivalved longitudinally and the rubber merely stretched across the divided half shells.

This required only a thicker stronger outer shell and made the construction simpler as the rubber could be assembled at less than full stretch. A new Loctite Brand 406 instant acrylic cement also made the construction practical.





The bivalved device in the evacuated condition (Rubber against the outer wall) is simply assembled and clamped over the thigh without any pressure on the leg, and can be left in place until required. Special valves and fittings were developed to operate the system. An electronic control to trigger on the QRS of the electrocardiogram with appropriate delays was assembled with appropriate monitoring system.

Test runs on the investigator with arterial pulse recorded by optical ear plethysmography demonstrated a clear augmentation of the diastolic run-off pressure following the dicrotic notch and compensatory slowing of heart rate. A useful technique for converting cylindrical tubing into two hemisections of tapered tubing was evolved that reduced the expense of making tapered half shells.











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